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(71) Applicant *(for all designated States except US)*: PLANT BIOSCIENCE LIMITED [GB/GB]; Norwich Research Park, Colney Lane, Norwich, Norfolk NR4 7UH (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BAULCOMBE, David, Charles [GB/GB]; 63 Caernarvon Road, Norwich, Norfolk NR2 3HY (GB). VOINNET, Olivier [FR/GB]; 21 Abinger Way, Eaton, Norwich, Norfolk NR4 6LJ (GB). LEDERER, Carsten, Werner [DE/DE]; In der Münchwiese 8, D–56566 Neuwied (DE).

(74) Agents: SIMON, Kremer et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

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(54) Title: GENE SILENCING MATERIALS AND METHODS

(57) Abstract

Disclosed are methods for silencing a target nucleotide sequence (preferably representing one or more endogenous genes, preferably in a systemic fashion) which is present in a first part of the plant, which method comprises transiently introducing into the cytoplasm of a cell in a second part of the plant, which cell comprises a nucleic acid encoding the target sequence and which is remote from said first part of the plant, a nucleic acid construct.

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GENE SILENCING MATERIALS AND METHODS

TECHNICAL FIELD

The present invention relates to methods and materials for controlling gene silencing in plants, and various processes and products employing these methods and materials.

PRIOR ART

Co-suppression and anti-sense suppression of endogenous genes

It is known that stably-integrated transgenes (referred to as 'STgenes' or 'intGENES' below) which may be constitutively expressed may be used to suppress homologous endogenous ('HEgenes') plant genes. This was discovered originally when chalcone synthase transgenes in petunia caused suppression of the endogenous chalcone synthase genes. Subsequently it has been described how many, if not all plant genes can be "silenced" by transgenes. Gene silencing requires sequence homology between the transgene and the gene that becomes silenced (Matzke, M. A. and Matzke, A. J. M. (1995), *Trends in Genetics*, 11: 1-3). This sequence homology may involve promoter regions or coding regions of the silenced gene (Matzke, M. A. and Matzke, A. J. M. (1993) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 44: 53-76, Vaucheret, H. (1993) *C. R. Acad. Sci. Paris*, 316: 1471-1483, Vaucheret, H. (1994), *C. R. Acad. Sci. Paris*, 317: 310-323, Baulcombe, D. C. and English, J. J. (1996), Current Opinion In Biotechnology, 7: 173-180, Park, Y-D., et al (1996), *Plant J.*, 9: 183-194).

When coding regions are involved, the transgene able to cause gene silencing may have been constructed with a

promoter that would transcribe either the sense or the antisense orientation of the coding sequence RNA. In at least one example the coding sequence transgene was constructed without a promoter (Van Blokland, R., et al (1994), *Plant J.*, 6: 861-877).

Co-suppression of transgenes

It has also become clear that gs can account for some characteristics of transgenic plants that are not easily reconciled with conventional understanding of genetics. For example the wide variation in STgene expression between sibling lines with a STgene construct is due in part to gene silencing: low expressers are those with a high level of gene silencing whereas high expressers are those in which gene silencing is absent or induced late in plant development. In this case there is no requirement for there to be an HEgene corresponding to the STgene (see e.g. Elmayan & Vaucheret (1996) *Plant J.*, 9: 787-797.

Viral resistance

In addition to observations of STgenes, gs has also been implicated in virus resistance. In these cases various factors including ectopic DNA interactions[6], DNA methylation[7], transgene expression level[8] and double stranded RNA[9] have been proposed as initiators of gene silencing.

Additionally in non-transgenic plants, it has been demonstrated that leaves which develop subsequently to systematic spread of a virus in a plant contain lower levels of virus than do symptomatic leaves. This resistance may be similar in nature to transgene-induced gene silencing (see e.g. Ratcliff et al (1997) *Science*, 276: 1558-1560).

Cytoplasmically replicating viral constructs

Biosource Technologies, in WO 95/34668, have suggested the use of genetic constructions based on RNA viruses which replicate in the cytoplasm of cells to provide inhibitory RNA, either anti-sense or sense ("co-suppressor") RNA. The constructs were used to inhibit a particular HEgene (phytoene desaturase). Cells were transfected with the cytoplasmically-replicating genetic constructions in which the RNA encoding region is specific for the gene of interest. The hybrid viruses spread throughout the plant, including the non-inoculated upper leaves (as verified by transmission electron microscopy). System-wide gene silencing was reported following transfection.

GB patent application 9703146.2, and PCT/GB98/00442, both filed in the name of John Innes Centre Innovations Limited, are hereby incorporated by reference. These applications, which were not published prior to the claimed priority date of the present application, discuss various constructs ('amplicons') which are intended to be stably integrated into the plant genome, and to generate cytoplasmically replicating constructs which are capable of eliciting gene silencing.

Silencing in animals

Fire et al (1998) Nature 391: 806-811 (not published prior to the claimed priority date of the present application) discusses the use of RNA, particularly double-stranded RNA, to achieve silencing of endogenous genes and GFP-transgenes in nematodes. The demonstrated interference effect was apparently able to cross cell-boundaries.

Applications for gene-silencing

In principle there is an enormous practical potential of gs for crop improvement. It is possible to silence genes conferring unwanted traits in the plant by transformation with transgene constructs containing elements of these genes. Examples of this type of application include gs of ripening specific genes in tomato to improve processing and handling characteristics of the harvested fruit; gs of genes involved in pollen formation so that breeders can reproducibly generate male sterile plants for the production of F1 hybrids; gs of genes involved in lignin biosynthesis to improve the quality of paper pulp made from vegetative tissue of the plant; gene silencing of genes involved in flower pigment production to produce novel flower colours; gene silencing of genes involved in regulatory pathways controlling development or environmental responses to produce plants with novel growth habit or (for example) disease resistance; elimination of toxic secondary metabolites by gene silencing of genes required for toxin production.

Gene silencing is also useful for investigating gene function in that it can be used to impose an intermediate or a null phenotype for a particular gene, which can provide information about the function of that gene *in vivo*.

A major complication in the practical exploitation of this phenomenon to date is the unpredictable and low occurrence of gene silencing. Therefore, it has not been realistic to attempt gene silencing in plants that are difficult to transform and for which it is difficult to produce many transformants. Similarly, it would be difficult to activate (and deactivate) gene silencing against several different traits or against several viruses in the same plant. Even with plants that are easy to transform the need to generate multiple lines limits the ease of exploitation of gene silencing.

INVENTION

The present inventors have now demonstrated a novel means of providing consistent, controlled, systemic gene silencing within a system, particularly a mature plant, which may (but is preferably not) a transgenic plant. This novel approach is clearly distinct from previously described approaches to gene silencing, for example, transwitch and antisense technologies, in that it describes a multicomponent system in which there is the potential to regulate the gene silencing spatially and optionally temporally.

The current invention is also distinct from the virus-induced gene silencing described previously by Biosource Technologies. In the current invention there is no absolute requirement that the transgenes conferring the gene silencing or their transcripts are able to replicate using viral components or through mechanisms that resemble virus replication, although in certain advantageous embodiments they may do so. Importantly, the systemic silencing of the invention does not require that the transgenes or their transcripts use virus-derived mechanisms to move between cells (e.g. 'movement proteins' as they are termed in the art).

These movement proteins are encoded by most (probably nearly all) plant viruses. Movement proteins are normally recognised by mutation analysis of a viral genome. Mutation of a movement protein gene affects the ability of a virus to spread in the infected plant but does not affect the ability of the virus to replicate. Examples of viral movement proteins identified in this way include the 30kDa protein of tobacco mosaic virus (Deom et al., 1987), the 25kDa, 12kDa and 8kDa triple gene block proteins of potato virus X (Figure 1C) (Angell

and Baulcombe, 1995; Angell et al., 1996; Verchot et al., 1998) and the tubule-forming protein of cowpea mosaic virus (van Lent et al., 1991). Some viruses also encode movement proteins specifically for translocation of the virus through the phloem of the plant. Examples of these long distance movement proteins include the 2b protein encoded in cucumber mosaic virus (Ding et al., 1995) and the 19kDa protein of tomato bushy stunt virus (Scholthof et al., 1995).

Until recently it has been considered that movement proteins open channels between plant cells and thereby mediate virus movement (Wolf et al., 1989). However it is now apparent that at least some of these proteins may also promote movement by suppression of a defence mechanism in the plant that blocks virus movement, which may itself be related to the gene silencing referred to hereinbefore. From these new findings, which are consistent with observations by Anandalakshmi et al. (1998) and Brigneti et al. (1998) [both in press] it is clear that movement proteins may be suppressors of gene silencing. Similarly the work of the present inventors suggests that certain proteins previously described only as pathogenicity proteins may also have a role in suppressing a gene silencing signal.

Thus it can be appreciated that stronger, systemic, gene silencing is obtained if transgene constructs for gene silencing do not also lead to expression of gene silencing by viral movement proteins or pathogenicity proteins, which are a fundamental part of the prior art systems which rely on the activity of vectors based on RNA-viruses. Such systems may be incapable of mediating a TIGS effect (see e.g. Dougherty, W.G, *et al* Molecular Plant-Microbe Interactions, 1994: 7, 544-552).

The novel gene silencing system of this invention was first demonstrated using transgenic N. benthamiana stably transformed with stably transformed with the gene for green fluorescent protein (designated stGFP).

The workers demonstrated that the expression of stGFP could be silenced by the transient presence of a GFP reporter gene (which was designated trGFP to distinguish it from the stGFP) using strains of *Agrobacterium tumefaciens* carrying binary Ti plasmid vectors or using direct infiltration. The silencing was systemic in nature, occurring remotely from the sites of infection or infiltration.

This approach has suggested the existence of a previously unknown signalling mechanism in plants that mediates systemic gene silencing. The signal of silencing is gene-specific and likely to be a nucleic acid that moves between cells.

A systemic, sequence-specific signal of gene silencing which is initiated by the transient presence (not stable integration) in part of a plant of foreign initiator nucleic acid or nucleic acid complex (termed hereinafter 'fiNA') which need not be capable of autonomous replication in the cytoplasm of a plant cell or movement from cell to cell, but which generates a signal which may be propagated systemically is an entirely novel and unexpected concept in plant biology. The observation has a number of important (industrially applicable) properties. These properties, and the characteristics of the fiNA required achieve them, will be discussed in more detail hereinafter.

The work of the present inventors, with hindsight, is consistent with data from other published experimental

systems and could be a general feature of gene silencing in plants.

Thus transgenic petunia exhibiting transgene-induced silencing of the genes required for flower pigment biosynthesis were shown to exhibit unusual and irregular patterns of pigmentation. These can perhaps be explained by an extracellular signal rather than by cell lineage-dependent cues of gene silencing (see Jorgensen (1995) *Science* 268, 686-691). It should be stressed that in that work the gene silencing of an HEgene (CHS) was induced in the test plants using a chimeric STgene. Although the paper speculates about a 2 state system of gene silencing, no information is given about how to switch gene silencing on.

Work by a different group demonstrated chitinase gene silencing in non-clonal sectors of transgenic tobacco (see Kunz et al (1996) *Plant J.* 10, 4337-450.). This work demonstrated both the 'self' inactivation of the expression of STgenes alone, plus inactivation of HEgenes by STgenes. The work also suggested that gene silencing was a post-transcriptional event. It was demonstrated that gene silencing occurred stochastically in progeny of transgenic plants but that 'resetting' to the non-silenced state occurred non-stochastically in developing seeds. These observations, plus the variegated pattern of silencing shown by some plants, demonstrated that the gene silencing phenotype was not merely a lineage event, but also highlighted the unpredictability of gene silencing. There is no suggestion in the paper of the use of fiNA to control gene silencing in non-silenced or 'reset' genes.

Palaqui et al, in The EMBO Journal (1997) V 16 No 15: pg 4738, demonstrated that grafting non-silenced scions onto gs-stock (co-suppressed ST and HE nitrate reductase

genes,) imposes silencing on the scion. The scion had to contain the STgene, and the silencing was unidirectional and could occur through a wild-type stem 'barrier' in which HE nitrate reductase genes are present and function as signal transducing resident genes. Although a diffusible messenger is postulated, there is no mention of generating or employing this messenger other than by the use of grafts of already-silenced homozygous plant stock.

The systemic signal demonstrated by the present inventors is also consistent with recent findings that gene silencing is associated with induced natural defence against viruses. The signal could move in the plant ahead of the inducing virus so that anti-viral gene silencing could delay spread of the infection front (Ratcliff et al (1997) *Science*, 276: 1558-1560). The data below also suggests that in certain situations, viral proteins may act to inhibit this signal propagation.

The provision of the signalling mechanism and the novel means by which it can be activated (transient presence of fiNA) opens up a number of possibilities which will be discussed in more detail hereinafter; essentially the ability to conveniently control gene silencing systemically will be useful both in the investigation of gene function, and the production of gene silencing plants, as well as in the investigation of the mechanisms of gene silencing.

Particularly useful is the ability to rapidly and consistently impose, at will, gene silencing on HE or STgenes of known or unknown function in order to investigate their phenotype.

Although the systemic signal is not yet structurally

characterised, a number of points about it can be made in the light of the present work. It is produced when fiNA is introduced in to a plant cell, particularly directly or indirectly into the cytoplasm, where the target gene or possibly a resident gene (as defined below) which is to be silenced is being transcribed, in the same plant cell, and there is sequence similarity between the coding regions of fiNA and target gene.

These findings suggest that a protein product, or the corresponding DNA or RNA, is a component of the signal. Of these, the protein product is the least plausible candidate because there is no mechanism known that explains how it could move systemically and specifically target the RNAs of the target. However, a nucleic acid-based signal could mediate sequence-specific gene silencing via a base-paired or triple helical structure with the target gene RNA (or the transcription product of homologous resident gene) as it moved between cells and tissues expressing that gene. Moreover, a nucleic acid could move in the plant, perhaps using the channels involved in virus or viroid movement. The demonstrated systemic spread of ST-GFP silencing (Fig. 2c) is consistent with this suggestion because it follows a course (Figs. 2c, 2g) that is similar to the pattern of virus spread in an infected plant.

Thus in a first aspect of the invention there is disclosed a method for silencing a target nucleotide sequence (e.g. a gene) in a plant comprising transiently introducing (i.e. not via a stably integrated transgene) into the cytoplasm of cells of that plant in which the target sequence is present (and preferably being transcribed) a foreign initiator nucleic acid (fiNA) which is:

(i) incapable of movement from cell to cell, and

(ii) optionally incapable of autonomous replication, and

(iii) has sequence homology with the gene to be silenced.

This method is used for silencing a target gene in a first part of a plant comprising the steps of:
(a) transiently exposing a second part of the plant, remote from said first part, to a foreign initiator nucleic acid (fiNA) as described above such as to generate a gene silencing signal,
(b) causing or allowing the signal to be propagated to the second part of the plant such as to silence said target gene.

"Causing or allowing" in this sense implies, in particular, that the construct giving rise to the fiNA (and hence signal) does not encode proteins which would block the signal e.g. movement proteins such as those which permit viral movment from cell to cell.

Thus the present inventors have demonstrated for the first time Transiently Induced Gene Silencing (or 'TIGS'). They have further demonstrated that a signal capable of propagating gene silencing can be initiated in a second part of the plant to induce silencing of a gene in the first.

Generally speaking, TIGS can be considered as having three phases:

(i) initiation of a gene silencing signal by the transient presence of fiNA in the cytoplasm of plant cells, which is described in more detail below,
(ii) translocation of a gene silencing signal (though not the fiNA itself) through tissues of the plant, which is facilitated by the expression of a HE gene or a ST gene with homology to the target gene in those tissues,
(iii) maintenance of the gene silencing signal within the cells of the plant, which may be remote from those which

were initially, transiently, exposed to the fiNA.

The various different features of TIGS will now be discussed in more detail:

"Silencing" in this context is used to refer to suppression of expression of the (target) gene. It does not necessarily imply reduction of transcription, because gene silencing is believed to operate in at least some cases post-transcriptionally. The degree of reduction may be so as to totally abolish production of the encoded gene product (yielding a null phenotype), but more generally the abolition of expression may be partial, with some degree of expression remaining (yielding an intermediate phenotype). The term should not therefore be taken to require complete "silencing" of expression. It is used herein where convenient because those skilled in the art well understand this.

The "systemic" silencing means that the target gene is silenced via a signal which is translocated substantially throughout the tissues of a plant (though certain tissues may not be silenced e.g. meristematic tissues, as discussed in more detail below).

The "target" gene (ie the gene to be silenced or the silenced gene) in the present invention may be any gene of interest. As discussed below it will share homology with the fiNA. In particular it may be a homologous endogenous gene (HEgene) or a stably transformed homologous transgene (STgene, as with the stGFP used above).

More than one target gene (e.g. a gene family) may be targeted simultaneously provided that they all share homology with the fiNA.

As will be discussed in more detail hereinafter, in certain aspects of the invention the identity or phenotype of the gene may be unknown - and indeed TIGS may be used to identify it.

The "fiNA", which may be either DNA or RNA, may be synthetic (ie man made) or naturally occurring nucleic acid sequence which is a homolog of the target gene or it may be a copy of all or part of the target gene in sense or antisense orientation. It may be double or single stranded, for instance it may consist of antisense (double stranded) RNAs.

It should be stressed that, unlike RNA viral-based vectors used to effect gene silencing in the art (e.g Biosource Technologies, in WO 95/34668) the fiNA itself lacks sequences which permit movement from plant cell to plant cell, and optionally allow replication in the cytoplasm of plant cells (i.e. fiNA need not be capable of autonomous replication in the cell).

Unlike the amplicons of PCT/GB98/00442 (which may optionally lack such movement sequences) fiNA is not generated by a stably integrated transgene in the plant.

Thus the crucial elements of the fiNA which give the potential for signal initiation are that:
(i) it is foreign to the plant, or is at least recognised as being foreign, possibly after interacting with existing nucleic acids in the plant,
(ii) it shares homology with all or part of the target gene (coding or non-coding strand),
(iii) it cannot move from plant cell to plant cell (more particularly, does not comprise sequence encoding movement proteins or other pathogenicity proteins which would interfere with the signal), and optionally it cannot replicate autonomously in plant cell cytoplasm.

The term "foreign" is used broadly to indicate that the fiNA has been introduced into the cells of the plant or an ancestor thereof, possibly using recombinant DNA technology, but in any case by human intervention. Put another way fiNA will be non-naturally occurring in cells in to which it is introduced. For instance the fiNA may comprise a coding sequence of or derived from a particular type of plant cell or species or variety of plant, or virus, placed within the context of a plant cell of a different type or species or variety of plant. Alternatively the fiNA may be derived from the plant genome but is present in "unnatural" cellular or chromosomal locations, or lacks certain features of the authentic endogenous sequence (gene or transcript). A further possibility is for the fiNA to be placed within a cell in which it or a homolog is found naturally, but wherein the fiNA is linked and/or adjacent to nucleic acid which does not occur naturally within the cell, or cells of that type or species or variety of plant, such as operably linked to one or more regulatory sequences, such as a promoter sequence, for control of expression.

Regarding the "homology" of the fiNA, the complete sequence corresponding to the transcribed sequence need not be used to effect gene silencing, as is clear from the prior art studies (which albeit did not use fiNA as described herein or provide TIGS). For example fragments of sufficient length may be used. It is a routine matter for the person skilled in the art to screen fragments of various sizes and from various parts of the coding or non-coding sequence of the target gene to optimise the level of gene silencing, for instance using systems based on the GFP system described later. It may be advantageous to include the initiating methionine ATG codon of the target gene, and perhaps one or more nucleotides upstream of the initiating codon. A further possibility is to target a conserved sequence within a

target gene, e.g. a sequence that is characteristic of one or more target genes in order to silence several genes which comprise the same or similar conserved sequence.

A fiNA may be 300 nucleotides or less, possibly about 200 nucleotides, or about 100 nucleotides. It may be possible to use oligonucleotides of much shorter lengths, 14-23 nucleotides. Longer fragments, and generally even longer than 300 nucleotides are preferable where possible if the fiNA is produced by transcription or if the short fragments are not protected from cytoplasmic nuclease activity.

It may be preferable that there is complete sequence identity between the fiNA and a relevant portion of the target sequence, although total complementarity or similarity of sequence is not essential. One or more nucleotides may differ in the targeting sequence from the target gene. Thus the fiNA of the present invention may correspond to the wild-type sequence of the target gene, or may be a mutant, derivative, variant or allele, by way of insertion, addition, deletion or substitution of one or more nucleotides, of such a sequence.

The fiNA need not include an open reading frame or specify an RNA that would be translatable. There may be a TIGS signal even where there is about 5%, 10%, 15%, 20% or 30% or more mismatch between the fiNA and the corresponding homologous target sequence. Sequence homology (or 'identity' or 'similarity' - the terms are used synonymously herein) may be assessed by any convenient method e.g. it may determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art.

Regarding translocation of the TIGS signal, as described

above this is generated when the cells of the plant are transiently exposed to the fiNA, and the translocating tissues comprise, and preferably transcribe (though not necessarily express) the target gene or another 'resident gene' sharing homology with the target gene and the fiNA for the gene silencing signal to be transmitted through such tissues. However it may not be necessary for all of the translocating tissues to transcribe the gene - as shown in the Examples below, the signal may be 'relayed' between expressing cells.

The resident gene, which is discussed in more detail below, may be either endogenous or exogenous to the plant. The term 'homology' in relation to the resident gene is used in the same way as it is used in relation to the fiNA/target gene above. In this case the crucial element is that the homology be sufficient to allow signal generation and/or propagation. As described above the homology will preferably be at least 70%, more preferably at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% or most preferably more than 95%.

The advantage of using an STgene as a resident gene is that its transcription may be more readily controlled (if desired) than a target gene which is an HEgene, as is discussed in more detail in relation to facilitating signal propagation below.

The "transient exposure" of the second part of the plant to the fiNA may be achieved by any convenient method. Essentially the fiNA should be introduced directly or indirectly (e.g. exposure of a fiNA produced in the nucleus from locally present foreign nucleic acid) into the cytoplasm of cells of the second part of the plant.

Known methods of introducing nucleic acid into plant

cells include use of a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 8721 (1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green *et al.* (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 290395, WO 8706614) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman *et al. Plant Cell Physiol.* 29: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* 87: 1228 (1990d) Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* 9: 1-11.

Preferably fiNA is introduced by microprojectile bombardment with gold particles. Vacuum infiltration or injection of agrobacterium or direct uptake mediated by carborundum powder, whiskers (see Frame et al, Plant J 1994, 6: 941-948) or electroporation.

Various embodiments will now be exemplified:

Introduction of fiNA - initiation of the signal

As described above fiNA may be introduced directly as naked DNA, or it may be transcribed from nucleic acid introduced into (but not stably integrated throughout) a plant. It should be stressed that although the fiNA must be located in the cytoplasm of the cell, there is no requirement that the fiNA be transcribed in the cell; thus there is no need for the fiNA to incorporate a promoter region in order to initiate the gene silencing signal or for it to be introduced into the cytoplasm via the nucleus.

In a further embodiment it may be possible to use a viral

or other extrachromosomal expression vector (which may or may not include translation signals) e.g. a viral-based vector, encoding the fiNA, and a replicase, but lacking transmissive elements (e.g. movement proteins or other pathenogenicity proteins) which could inhibit the generation of a signal which can move beyond the infected parts of the plant, or be sustained within the plant after initial introduction. However viruses, particularly those which are transmissible, may be undesirable for other reasons e.g. safety, resistance etc.

In a further embodiment it may be achieved by transiently (e.g. locally) initiating the transcription of a fiNA-encoding sequence which is present in the cells, possibly the nucleus or the genome, of the second part of the plant.

This may be achieved by the use of Ti-based binary vectors (cf. use of the trGFP described below). Generally speaking, those skilled in the art are well able to construct vectors and design protocols for transient recombinant gene transcription. For further details see, for example, *Molecular Cloning: a Laboratory Manual:* 2nd edition, Sambrook *et al*, 1989, Cold Spring Harbor Laboratory Press.

Optionally transcription of the fiNA may be placed under the control of an activating agent, for instance using an inducible promoter.

The term "inducible" as applied to a promoter is well understood by those skilled in the art. In essence, transcription under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of transcription (or no

transcription) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus.

One example of an inducible promoter is the GST-II-27 gene promoter which has been shown to be induced by certain chemical compounds which can be applied to growing plants. The promoter is functional in both monocotyledons and dicotyledons. It can therefore be used to control gene expression in a variety of genetically modified plants, including field crops such as canola, sunflower, tobacco, sugarbeet, cotton; cereals such as wheat, barley, rice, maize, sorghum; fruit such as tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, and melons; and vegetables such as carrot, lettuce, cabbage and onion. The GST-II-27 promoter is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues. Other example inducible promoters are well known to those skilled in the art, the choice of which will be determined by the convenience of using the inducing agent in the particular application being carried out.

Another suitable promoter may be the DEX promoter (Plant Journal (1997) 11: 605-612).

In this embodiment the activating agent can be applied locally to one or more regions of the plant in which the fiNA-encoding construct has been introduced (the 'second part') in order to achieve the remote silencing of other ('first part').

In a most preferred aspect, the fiNA may be introduced as a construct corresponding to a truncated 'amplicon' of GB

98/00442. This will generally comprise:
(i) a plant promoter
(ii) a nucleic acid sequence operably linked to that promoter, said sequence encoding an RNA-dependent replicase, and further encoding fiNA, which is itself operably linked to a sub-genomic promoter capable of being recognised by said replicase, such that the fiNA is capable of autonomous cytoplasmic replication,
with the proviso that the nucleic acid sequence does not encode active viral movement proteins (plus optionally pathogenicity proteins) which would otherwise inhibit the TIGS signal from spreading systemically in the plant into which the construct is introduced.

By "replicase" is meant, where appropriate, all the required components to give replicase function. The construct does not encode "active movement proteins" in the sense that, although a movement proteins may be encoded, they are not functional e.g. because one or more has been deleted or modified.

Propagation and maintenance of the signal through the plant

The advantage of achieving systemic gene silencing using transient activation or introduction of fiNA in a localised area (e.g. by application of a specific agent) is that there is no requirement for the inducing agent of fiNA to be translocated within the tissues of the plant or be applied to all parts of the plant. Once initiated the signal can induce gene silencing in remote parts of the plant. This gene silencing is stable and persists even after the fiNA ahs been removed.

By "remote" is meant the first and second parts of the plant are spatially separated, although obviously connected via the plant tissues. It may be advantageous

if the first part of the plant is above the level of the second, or if the route corresponds to the 'source-sink' movement of photosynthetic products from regions in which they are concentrated to regions of use. The observations described in the Examples suggest that signal movement mimics in some respects viral or viral-vector movement. It should be stressed, however, that neither the signal of the present invention, nor the fiNA used to initiate it, are viruses, for instance mobile, cytoplasmatically replicable vectors.

It should also be stressed that the part of the plant in which the target gene is to be silenced may encompass all, or almost all, of that part of the plant which is not directly exposed to the fiNA i.e. systemic silencing.

Thus in one embodiment of this aspect, the target gene is silenced systemically in the plant tissues i.e. in the first and second parts of the plant and the tissues between them, (cf. the stGFP described below).

It may not be necessary for all the cells in these tissues to transcribe the target gene, as detailed in the Examples.

Alternatively, some or all of the cells of the connecting plant tissues will comprise a resident gene, the transcription (though not necessarily expression) facilitates the propagation of the signal.

By "resident gene" is meant a gene (endogenous or exogenous) which is homologous to the target gene and homologous to the fiNA such as to facilitate transduction of the TIGS signal.

Thus in a second embodiment of this aspect, the target gene is transcribed only in a second, remote, part of the

plant (e.g. it is expressed in a tissue specific manner), but a resident gene which is homologous to the target gene is present and preferably transcribed in the plant tissues in the second part of the plant and/or the tissues between the first and second parts of the plant. Presence or preferably transcription of this resident gene may thus serve to cause or allow signal propagation.

This embodiment permits control of tissue specific target genes. The resident gene serves to assist systemic spread of the signal. The systemic spread of the signal can thus be controlled at an additional level to the direct control of the fiNA exposure, providing further temporal and spatial control over gene silencing:

By regulating the transcription of the resident gene in the cells carrying the TIGs signal, it will be possible to determine whether gene silencing in the first part of the plant is activated effectively, or to affect the tissue specificity of gene silencing.

Transcription of a resident (STgene) may be altered by use of an inducible promoter, such as is described above in relation to the fiNA.

It will be apparent from the foregoing that the invention embraces methods of controlling gene silencing in plants by manipulating the presence or transcription of the fiNA or the propagation of the signal. e.g. by controlling the presence or absence of an activating agent which induces transcription of a resident gene. Physical methods for manipulating the resident gene expression are also envisaged. For instance grafts of tissue between the different parts of the plant which are either permissive (i.e. contain cells having the resident gene) or non-permissive (cells don't have the resident gene) can be used to control translocation of the signal.

Selected applications for TIGS

In embodiments of the present invention which have been experimentally exemplified as described below for illustrative and non-limiting purposes only, the transiently introduced gene encoding the fiNA that determined the target of gene silencing was the gene encoding the jellyfish green fluorescent protein GFP (Chalfie *et al.* (1994) *Science* 263: 802-805). This was used to silence a stably integrated GFP transgene.

Any other ST- or HEgene of a plant, or STgene of animal, fungal, bacterial or viral origin may be a target gene provided that the fiNA contains a corresponding homologous sequence.

In one aspect of the present invention, the target gene may be of unknown phenotype, in which case the TIGS system may be employed to analyse the phenotype by generating a systemic (or widespread) null (or nearly null) phenotype.

Thus a further aspect of the invention comprises a method of characterising a target gene comprising the steps of:
(a) silencing the target gene in a part or at a certain development stage of the plant using the TIGS system described above,
(b) observing the phenotype of the part of the plant in which or when the target gene has been silenced.

Preferably the gene is silenced systemically. Generally the observation will be contrasted with a plant wherein the target gene is being expressed in order to characterise (i.e. establish one or more phenotypic characteristics of) the gene.

There are several advantages of the current method over

alternative methods in which the targeted gene is inactivated by insertional or other mutagenic procedures or in which gene silencing is uncontrolled. The advantage over mutagenic procedures applies when there is more than one homologous gene carrying out the role of the target gene. Mutagenic procedures will not normally reveal a phenotype in that situation. A second situation where the current invention has advantage over both mutagenic and unregulated gene silencing procedures applies when the target gene has a lethal phenotype. The controllable attribute of the gene silencing will allow the phenotype of such genes to be investigated and exploited more efficiently than using the alternative methods available prior to the disclosure of the current invention.

This aspect is particularly useful given the significant amount of sequence data currently being generated in genomics projects which is unassigned in terms of function or phenotype. Thus even if the gene exerts its effects only in particular tissues, this may be detectable without having to ensure that a virus has permeated the entire plant (as in Biosource Technologies, WO 95/34668).

Nor, for the identification of HE genes, would it be necessary to try and generate a transgenic plant in which gene silencing is already activated to observe the effect.

In a further aspect there is disclosed a method of altering the phenotype of a plant comprising use of the TIGS method.

Traits for which it may be desirable to change the phenotype include the following: colour; disease or pest resistance; ripening potential; male sterility.

For instance male sterile plants are required for production of hybrid seed. To propagate the male sterile lines it is necessary to restore male fertility. In the examples in which male sterility is induced by a transgene it would be possible to restore male fertility by controlled silencing of the transgene using the approach described above.

Many genes have multiple roles in development. They may be required, for example, in embryo development and in the development of organs or tissues in the mature plant. Obviously it would not be possible to silence these genes unless the silencing system could be controlled so that it is not active in embryo development. The system described here could be used to provide that control.

Other traits will occur to those skilled in the art. In each case the only necessity is that sufficient is known about the target gene(s) to devise suitable fiNA, which may of course be optimised without burden to achieve the desired effect. If the target gene is not expressed systemically, then it may be necessary to produce a transgenic plant wherein a resident STgene is transcribed systemically in order to allow signal propagation. The fiNA can then be used to initiate the signal.

The production of transgenic plants is now very well known to those skilled in the art, as evidenced by the various reported methods some of which are recorded in non-prior published GB patent application 9703146.2 in the name of John Innes Centre Innovations Limited, the content of which is incorporated herein by reference.

In a further aspect of the present invention there is disclosed a method for producing a systemic gene silencing signaling agent in a plant, which is capable of silencing a target gene comprising causing or allowing

the transient exposure of a part of the plant expressing said target gene or a homolog thereof to a fiNA.

The systemic gene silencing signaling agent is characterised in that it
(a) comprises nucleic acid,
(b) is capable of mediating sequence-specific gene silencing of a target gene,
(c) it is obtainable by transient exposure of a plant cell transcribing said target gene or a homolog thereof to a fiNA,
(d) is capable of moving between a first and second part of the plant, said parts being connected by cells comprising, and preferably transcribing said target gene or a homolog thereof, which movement is inhibited my movement or pathogenicity proteins as discussed above.

The various nucleic acids of the present invention may be provided isolated and/or purified (i.e. from their natural environment), in substantially pure or homogeneous form, or free or substantially free of other nucleic acid. Nucleic acid according to the present invention may be wholly or partially synthetic. The term "isolate" encompasses all these possibilities.

Also embraced by the present invention is a transgenic plant comprising a target gene which has been systemically silenced using TIGS.

The present invention may be used in plants such as crop plants, including cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorgum, millet, cassava, barley, pea and other root, tuber or seed crops. Important seed crops are oil seed rape, sugar beet, maize, sunflower, soybean and sorghum. Horticultural plants to which the present invention may be applied may include lettuce, endive and vegetable brassicas including cabbage,

broccoli and cauliflower, and carnations and geraniums. The present invention may be applied to tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper, chrysanthemum, poplar, eucalyptus and pine.

The present invention will now be illustrated and exemplified with reference to experimental results and the accompanying Figures. Further aspects and embodiments of the present invention, and modifications of those disclosed herein, will be apparent to those skilled in the art. All documents mentioned anywhere herein are incorporated by reference.

FIGURES

Figure 1. Transgene and Viral Constructs

a T-DNA from pBin-35S-mGFP5 used for *Nicotiana benthamiana* stable transformation (pnos: *nos* promoter, tnos: *nos* terminator, 35S: CaMV-35S promoter, RB: right border, LB: left border). This is the STgene construct.

b T-DNAs from various binary vectors carried by *Agrobacterium tumefaciens* strain LBA4404 used for leaf infiltrations (OCS: octopine synthase terminator, BaR: BASTA resistance gene). These are TRgene constructs. lacZ: multiple cloning site, inserted for cloning facilities.

c Structures of PVX-GUS[17] and PVX-GFP[16]. Expression of the inserted marker genes is controlled by a duplicated coat protein (CP) promoter (shaded boxes). Other abbreviations are RdRp: RNA dependent RNA polymerase, and 25K, 12K, 8K: cell-to-cell movement proteins. These constructs were used, inter alia, in determining whether gene silencing was pre- or post-transcriptional.

Figure 2. Expression of GUS and GFP reporter genes in *N. benthamiana*

These images were all produced under UV illumination except for the bottom panels of E and F and panels I-L that show leaves stained for GUS activity[24]. The method and abbreviations are described in more detail in Example 1. Depending on the exposure time and the source of UV, GFP appears green or yellow. In the absence of GFP the chlorophyllous plant tissue appears red.

(a) A leaf of a stably integrated GFP homogene (stGFP) plant

(b) A leaf of a non-transgenic (not) nt plant.

(c-d) stGFP plants infiltrated 18d previously with a culture of the NPT:GUS:GFP strain of *A. tumefaciens*, prepared in the presence (c) or in the absence (d) of acetosyringone; the arrows indicate the infiltrated leaves.

(e-f) Expression of trGFP (top panel) and GUS (bottom panel) in leaves of an nt plant (e) or an stGFP plant (f) that had been infiltrated with the NPT:GUS:GFP strain of *A. tumefaciens* 2 days previously. The arrow in (e) indicates the zone of stGFP suppression at the edge of the infiltrated zone where A line of red fluorescent tissue is observed.

(g) Close-up view of an axillary shoot emerging from one of the three fully expanded leaves of the plant presented in (c). Leaves on these axillary shoots always show very strong stGFP suppression. The diffuse patches of residual expression of stGFP fade when these leaves expand. Some of the smaller leaflets on the axillary shoots as shown in this panel (arrow) are uniformly red.

(h) UV illumination of upper leaves emerging from the main stem of A stGFP plant infiltrated 18 days previously with water (left), or with the NPT:GUS:GFP strain of *A. tumefaciens*. (middle and right).

(i) Leaves shown in (h) were stained for GUS activity.

(j) A leaf infiltrated with an NPT:GUS:GFP strain of *A.*

tumefaciens as an internal control for the histochemical GUS staining shown in (i).

(k-l). PVX-GUS foci observed on A systemic leaf of an stGFP plant infiltrated with either the NPT:GUS:GFP strain of *A. tumefaciens* (k) or with water (l). Leaves were inoculated with PVX-GUS and collected after 5 days for GUS staining. When leaves were collected later than 5 days post-inoculation, the GUS foci had spread to the veins, indicating a potential for systemic spread of PVX-GUS independently of stGFP silencing.

Figure 3. Northern analysis of stGFP and PVX-GFP RNA. stGFP plants(GFP) or nt plants (NT) were infiltrated with either water (Mock), or the NPT:GUS:GFP strain of *A. tumefaciens* previously induced with acetosyringone (N:G:G)-X(N:G:G-) indicates that the culture was not previously induced. After 20 d, two upper leaves were inoculated with water (Mock) or PVX-GFP. 5d after virus inoculation, total RNA was extracted from one of the two inoculated leaves and northern analysis on 10μg of RNA was carried out to detect accumulation of the stGFP RNA and PVX-GFP RNA (indicated on the left side of the upper panel). The heterodisperse RNA species in tracks 9-14 represent sub-genomic and degraded RNA species and are typical of PVX RNA samples of inoculated leaves. The lower panel shows probing of the northern blot with an rRNA probe to confirm equal loadings of RNA.

In Figure legends 4 to 7, the intGFP refers to stably integrated GFP, while epiGFP refers to infiltrated sequence.

Figure 4. Constructs used in Example 13

The T-DNA constructs used for Agrobacterium infiltrations are derived from the N:G:G construct. The 35S promoter controlling the GFP gene has been replaced by the nos

promoter in the N:Gnos construct, and has been deleted in the N:GΔ construct.

Figure 5. Kinetics of translocation of the TIGS signal

The top diagram illustrates the order of events described below. One leaf of intGFP plant was infiltrated with the N:G:G strain of A. tumefaciens (previously induced with acetosyringone), and subsequently removed 1,2,3,4 or 5 days after infiltration. The percentage of plants undergoing TIGS after removal of the infiltrated leaf was then assessed under UV illumination. Each dot on the diagram represents the average percentage obtained from 30 individual plants infiltrated at the same time (see Example 14).

Figure 6. Biolistic activation of TIGS

(A) DNA constructs tested for biolistic activation of TIGS. The pUC35S-GFP plasmid contains the 35S-GFP expression cassette from pBin35S-GFP (Figure 1). The GFP plasmid contains only the full-length GFP open reading frame from pBin35S-GFP cloned as a BamHI-SalI restriction fragment in pUC19. The ..P and G.. DNA constructs are linear, PCR-amplified fragments of the GFP open reading frame and are respectively 348 and 453 bp long. Equal amounts of each construct were bombarded (see Experimental Procedures and Example 16).

(B) Effect of the length of homology between epiGFP and intGFP on biolistic activation of TIGS. The intGFP seedlings were bombarded with a series of PCR-amplified fragments sharing a similar physical length but harbouring 3' terminal fragments of GFP cDNA of varying length. These fragments were amplified from a pBluescript vector containing the full-length GFP open reading frame by using one vector-specific primer and one GFP-specific

primer. The white dot on the diagram represents the 5′ end of the GFP open reading frame. Equal amounts of each construct were bombarded (see Experimental Procedures, and Example 16).

Figure 7. TIGS requires an interaction of epiGFP and intGFP

See Example 17.

(A) Bombarded epiGFP and inoculated viral constructs. The ..P and GF. DNA constructs are derivatives of the GFP construct described in Figure 5A. PVX-GF and PVX-P are PVX vectors carrying the GF. and ..P restriction fragments of the GFP open reading frame, respectively.

(B) Northern analysis of intGFP and PVX-GF/GFP RNAs. The top diagram illustrates the order of events described below. First intGFP seedlings or non-transformed plants (NT) were bombarded with either uncoated gold particles (-) or gold particles coated with either the GFP or the ..P construct. After 21 days, two upper leaves were inoculated with either water (Mock), PVX-GFP or PVX-GF. The plants bombarded with GFP or derivatives exhibiting TIGS were selected for the virus inoculation. Five days after virus inoculation, total RNA was extracted from one of the two inoculated upper leaves and Northern analysis of 10 (g of RNA was carried out to detect accumulation of the intGFP and PVX-GF/GFP RNA (indicated on the left side of the upper panel).

Figure 8: pPVX209 and pPVX210A

As described in Example 19, the CP was deleted from pPVX209 [Fig 8(a)] to create pPVX210A [Fig 8(b)]. The sequence is numbered from the 35S promoter, with the *Sac*I site immediately upstream of the promoter being numbered

as nucleotide 4.

Figure 9: pCL-vectors and progenitor construct

After eliminating the TGB (triple gene block) tagged PCR fragments amplified from pPVX210A were re-inserted to restore replicase function. Shown are (a) pCL100; (b) pCL101; (c) pCL102; (d) pCL105 (includes a 1729 bp deletion in the replicase); (e) pCL106 (includes a PCR fragment from pPVX210A to restore GFP function and enhance the production of sub genomic RNA); (f) progenitor construct pA500 [see Table 2; Example 19; (g) pCL103; (h) pCL104. See Figure 8 for explanation of terms.

Figure 10: Insertion of pUC19 constructs into plasmid pSLJ755/5.

Numbers in pSLJ755/5 are relative to the *Sac*I cloning site.

Figure 11: Positive strand sequences of constructs

Restriction sites used in cloning are underlined and labelled in grey. '*Xxxx*' indicates the ligated *Sal*I/*Xho*I hlaf sites. Abridged parts of the sequences are labelled in tildes ('~'). The 144 underlined bases represent the duplicated CP promoter region which together with the downstream GFP 5' end was inserted into pCL100 to create pCL106. Bases in lower case indicate non-viral sequence introduced by PCR primers used in cloning. Sequences confirmed after the respective cloning step are double underlinded, single bp exchanges or deviations not unambiguously falsified by examining the sequencing raw data are in minor case italics. Spacing for the CP deletion is condensed in TGB deletion contructs.

(a) pPVX209 (10762 nt)
(b) pPVX210A (10024 nt)
(c) pCL100 (8753 nt)
(d) pCL102 (8918 nt)
(e) pCL101 (8780 nt)
(f) pCL106 (8901 nt)

EXAMPLES

General Methods - Examples 1 to 12

Plant transformation.

Four independent lines of *Nicotiana benthamiana* plants carrying the GFP transgene (stGFP plants) were generated by the *A. tumefaciens*-mediated leaf disk transformation method[22]. For transformation, we used the disarmed *Agrobacterium* strain GV-3101 containing the binary vector pBin-35S-mGFP5[23]. Restriction digestion and Southern analysis showed that each line harbours a single T-DNA integration site, consistent with the observed 3:1 segregation of the expression of GFP in the R1 generation. In all cases, this single locus is associated with one intact copy of the GFP transgene. Northern analysis showed comparable high levels of GFP mRNA in these four independent lines. All stGFP plants used in this work were homozygous, selfed F1 progeny of the primary transformants.

Infiltration of Agrobacterium and the selective enrichment assay

Infiltration of Agrobacterium cultures for transient expression was based on a previously-described method[13]. First, the constructs shown in Figure 1b were transferred to *A.tumefaciens* GV3101 by triparental mating and the strains were plated on minA medium. A single colony was

inoculated into 5 ml LB medium supplemented with the appropriate antibiotics, and grown at 28°C for 48 hours. One ml of the culture was transferred to 100 ml LB with 10 mM MES pH 5.6 and 20 μM acetosyringone, and grown at 28°C for 16 hours. The bacteria (OD600=1) were spun down, suspended in 50 ml 10 mM $MgCl_2$ and kept at room temperature for 3 hours. The infiltration, performed with a 2 ml syringe, was to one or two expanded leaves of 3 week-old seedlings. The infiltrated leaves were then sealed in a small plastic bag for two days. Seedlings were maintained in A glasshouse between 20°C and 25°C. Artificial illumination was used, if necessary, to provide A day length of 16 hours or more.

The selective enrichment assay for *Agrobacterium* was as described[19]. Using this procedure a single isolated *Agrobacterium* cell mixed with 0.1 g of tobacco tissue could be enriched to the late exponential phase after 3 days of incubation.

General procedures.

PVX-GFP and PVX-GUS inocula were sap extracts of plants (*Nicotiana clevelandii*) infected with *in vitro* transcripts of the corresponding cDNA clones[16,17]. RNA isolation and Northern analysis were done as described[17]. The probe used for hybridization was a ^{32}P-labelled cDNA corresponding to the entire GFP open reading frame. Histochemical staining of plant material for GUS activity was performed according to the method of Jefferson[24].

General Methods - Examples 13-19

These were as above except:

Infiltration of Agrobacterium.

Infiltration of A. tumefaciens was based on a previously-described method (English et al., 1997). The constructs shown in Figure 4 were transferred to A. tumefaciens (strain GV3101, unless otherwise stated) by triparental mating or electroporation and the strains were plated on minA medium. Procedure was as described above.

Grafting procedure

Non-transformed and transgenic N. benthamiana plants were grown about 1 month before grafting. The stocks were beheaded 10-15 cm from the soil and wedge-grafting was performed with scions of similar age. The graft junction was then fastened and protected from desiccation by Parafilm. During the first week after grafting, plants were covered with a plastic bag to maintain high humidity conditions.

Seedling bombardment

N. benthamiana seeds were sterilised with 0.25% sodium hypochlorite for 15 min and rinsed 3 times with sterile water. Seeds were germinated for 7-10 days on MSR6 medium. One day before bombardment the seedlings in groups of 10-12 were transferred onto fresh MSR6 medium distributed over a 3.2 cm2 target area. DNA coating and particle bombardment were carried out as described previously (Christou et al., 1991). Each group of 10 seedlings was bombarded twice with 163ml of gold particles coated with 326 ng of DNA and accelerated at 12 Kv. Two weeks after bombardment seedlings were transferred to a glasshouse between 20°C and 25°C. Artificial illumination was used, if necessary, to provide a day length of 16 hours or more.

In vitro propagation

N. benthamiana leaves were harvested from greenhouse-grown plants. Leaves were sterilised with 0.25% (w/v) sodium hypochlorite for five minutes and rinsed three times with sterile distilled water. Leaf disks were aseptically plated onto MSR6 medium (Vain et al., 1998) complemented with 1 mg/l 6-Benzylaminopurine and 0.1 mg/l (-Naphthaleneacetic acid. Culture was conducted in 2 cm deep Petri dish sealed with Micropore(tape, at 23(C and under a 16 hours photoperiod. Leaves were subsequently transferred at 15 day intervals onto fresh medium. After 4 to 6 weeks the regenerated shoots were dissected and rooted onto MSR6 medium.

GFP imaging

Visual detection of GFP fluorescence in whole plant was performed using a 100 W hand-held long-wave ultraviolet lamp (UV products, Upland CA 91786, Black Ray model B 100AP). Plants were photographed with a Kodak Ektachrome Panther (400 ASA) film through a Wratten 8 filter. Exposure times varied up to 70 sec depending on the intensity of the fluorescence and the distance of the camera and lamp from the plant. Observation of explants cultured in vitro was carried out using a MZ12 Leica dissecting microscope coupled to an epifluorescent module. Photographs were taken using Kodak Ektachrome Panther (400 ASA) film. Confocal microscopy was performed under a Leica DMR module coupled to a Leica TCS-NT system. A 100 mW Argon ion laser was used to produce blue excitation light at 488 nm (emission filter 522 nm). Using these filter combinations, background autofluorescence from the samples was removed. Individual images were stored on optical disc.

Construction of PVX derivatives and in vitro transcription

PVX-GFP has been described previously (Baulcombe et al., 1995). PVX-GF was made by replacing the original GFP insert in the PVX vector pTXS-GFP (Baulcombe et al., 1995) by the mGFP5 insert from pBin-35S-mGFP5 (Haseloff et al., 1997) and by removing the 354 bp fragment between a ClaI site (position 465 within the GFP5 coding sequence) and a SalI site at the 3' end of GFP5 (position 818). PVX-P was made by inserting a ClaI-SalI restriction fragment from GFP5 into the PVX vector pP2C2S (Baulcombe et al., 1995). Viral inocula were sap extracts of plants (N. clevelandii) infected with in vitro transcripts (Chapman et al., 1992) of the corresponding cDNA clones.

Agroinfiltrated and bombarded epiGFP constructs

The N:G:G binary vector (Figure 1) is based on pBIN 35S:GFP4 (Haseloff et al., 1997) in which the LacZ polylinker from pUC19 has been inserted in the HindIII blunted restriction site located upstream the 35S promoter of GFP4. A 35S-GUS expression cassette from pSLJ4D4 (Jones et al., 1992) was then inserted in the LacZ polylinker as a HindIII-EcoRI restriction fragment. The N:Gnos and N:GΔ constructs (Figure 4) are derived from pBin 35S:GFP4. N:GΔ was obtained by removal of the 35S promoter of GFP4 by a BamHI-HindIII restriction, followed by blunt ending (Klenow) and relegation. N:Gnos was obtained by removal of the 35S promoter by a BamHI-HindIII restriction, followed by Klenow DNA filling and insertion of the nos promoter. The pUC35S-GFP construct (Figure 6) was obtained by inserting the 35S:GFP4 expression cassette from pBIN 35S:GFP4 (HindIII-EcoRI restriction fragment) in pUC19 . The GFP construct was obtained by inserting the full-length GFP open reading frame from pBIN 35S:GFP4 (BamHI-SacI restriction fragment) in pUC19 (Yanisch-Perron et al., 1985). The "G.." fragment (Figure 6) was PCR-amplified from pBIN 35S:GFP5 (Haseloff et al., 1997) using primers

GGATCCAAGGAGATATAACAA and AAATCGATTCCCTTAAGCTCG (pos1 and pos453 in the GFP5 cDNA, respectively). The "..P" fragment (Figure 6) was PCR-amplified from pBIN 35S:GFP5 using primers AGCTTAAGGGAATCGAT and CTTAGAGTTCGTCATGTTTGT (pos454 and pos813 in the GFP5 cDNA, respectively). The series of PCR-amplified fragments used for the study of the effect of the length of homology between epiGFP and intGFP (Figure 6B) was obtained from pBluescript in which the complete GFP5 cDNA was inserted as a BamHI-SacI restriction fragment. Primer combinations used for each amplification are:
(AGCTTAAGGGAATCGAT-TTGTGGCCGAGGATGTTT);
(AAATCGATCCCTTAAGCTCG-GGGTAACGCCAGGGTTTTCC);
(AGTAGTGACAAGTGTTGGCC-AGCGGGCGCTAGGGCGCT);
(TGACAGAAAATTTGTGCCCATT-GTAAAGCACTAAATCGGAACC);
(TTGGGACAACTCCAGTGAAAA- CCACTACGTGAACCATCAC).
The ...P and GF. constructs are respectively linear ClaI-SalI and BamHI-ClaI restriction fragments from the GFP construct described above.

General procedures

RNA isolation and Northern analysis were done as described (Mueller et al., 1995). The probe used for hybridisation was a 32P-labelled cDNA corresponding to the entire GFP open reading frame. Histochemical staining of plant material for GUS activity was performed using standard procedures (Jefferson, 1987).

Example 1: The gene silencing signal imposes remote silencing

To develop a reproducible system for activation of gene silencing we have used transient expression of silencer transgenes in *Nicotiana benthamiana*. The target of gene silencing (Fig. 1a) in these experiments encodes the jellyfish green fluorescent protein (GFP)[11] that can be

monitored non-invasively: leaves of transgenic GFP plants appear green under UV light (Fig. 2a) whereas non transgenic (nt) leaves appear red due to chlorophyll fluorescence (Fig. 2b). To deliver silencer transgenes, we infiltrated leaves[12,13] of *N. benthamiana* with strains of *Agrobacterium tumefaciens* carrying various binary Ti plasmid vectors (Fig. 1b), including one with a GFP reporter gene. We refer to the stably integrated and transiently expressed GFP transgenes as stGFP and trGFP, respectively.

At 2 days post-infiltration with the NPT:GUS:GFP strain of *A. tumefaciens* (Fig. 1B) there was expression of both the GUS and the trGFP reporter genes in the infiltrated tissues (Figs. 2e, 2f). In the stGFP transgenic lines (Fig. 2f) the strong green fluorescence due to the trGFP was superimposed over a weaker background fluorescence from the stGFP. However, at the edge of the infiltrated zone there was a thin line of red fluorescent tissue (Fig. 2f) indicating that stGFP expression had been suppressed.

Although the zone of stGFP suppression did not spread further within the infiltrated leaf, by 18 days post-infiltration there was suppression of stGFP in the upper leaves (Fig. 2c) of the NPT:GUS:GFP infiltrated plant. This effect was most pronounced in the stem and leaves that were directly above the infiltrated leaf and in the tissues surrounding the veins (Fig. 2c, 2h). In leaves of the axillary shoots (Fig. 2g) and in some uppermost leaves (Fig. 2h) there was complete suppression of green fluorescence due to stGFP. The time-course of stGFP suppression and its pattern of spread through the vegetative parts of the infiltrated plants were consistently observed in 5 independent experiments involving 20 plants of each of 4 independent stGFP lines.

Example 2: The gene silencing signal is sequence specific

There was no suppression of stGFP when the plants were infiltrated with the NPT:GUS, GUS:BAR or empty vector strains of *A. tumefaciens* (Fig. 1b). If the suppression had been caused by the infiltration process these control strains would have caused suppression of stGFP. Similarly, if the 35S promoter or nos terminator components of the trGFP are involved, there would have been suppression of stGFP following infiltration with the NPT:GUS and GUS:BAR strains (Fig. 1b): these constructs have both 35S promoters and nos terminators. Therefore, the systemic suppression of stGFP is a sequence-specific effect based on the common presence of GFP coding sequences in stGFP and trGFP.

Example 3: The gene silencing signal requires uptake of the transgene coding for the fiNA

The *A. tumefaciens* cultures used in these experiments contained acetosyringone as an inducer of virulence (Vir) functions[14]. In the absence of Vir gene expression there is no transfer of T-DNA (between the right and left borders; Fig. 1b) from the Ti plasmid into the plant cell. Consequently, when leaves of nt *N. benthamiana* were infiltrated with the NPT:GUS:GFP strain of *A. tumefaciens* incubated without acetosyringone, there was no expression of GUS or trGFP at 2 days post-infiltration. In addition, there was no systemic suppression of stGFP by 18 days post-infiltration (Fig. 2b, 2 days). From this result we conclude that the systemic suppression of stGFP requires T-DNA-mediated transfer of trGFP nucleic acid into plant cells.

Example 4: The gene silencing signal effects post-transcriptional silencing

In the tissue exhibiting the systemic suppression of stGFP, the steady state levels of stGFP RNA were reduced below the level of northern blot detection (Fig. 3 lanes 1-4) indicating that there is gene silencing. To determine whether the mechanism of stGFP silencing is transcriptional or post-transcriptional, we exploited previous demonstrations that post-transcriptionally silenced transgenes confer resistance against modified potato virus X (PVX) constructs in which there is sequence similarity to the silencer transgene[15]. A transgene exhibiting transcriptional gene silencing did not affect the corresponding viral construct[15]. The modified PVX in the present analyses (Fig. 1c) carried either a GFP or a GUS reporter gene (PVX-GFP[16] and PVX-GUS[17] respectively). The viral inocula were applied to the upper leaves of *N. benthamiana* at 18d post-infiltration with either water or cultures of *A.tumefaciens*.

Northern analysis (Fig. 3) revealed that at 5 days post-inoculation there was abundant PVX-GFP RNA in leaves of nt and stGFP *N. benthamiana* that had been previously infiltrated with water (Fig. 3, lanes 11-13). The PVX-GFP RNA was also abundant if the plants had been previously infiltrated with the NPT:GUS:GFP strain prepared in the presence (nt line) or absence (stGFP line) of acetosyringone (Fig. 3, lanes 9,10,14). However, in the stGFP-silenced leaves of plants that had been previously infiltrated with the acetosyringone-treated NPT:GUS:GFP strain of *A.tumefaciens*, the accumulation of PVX-GFP RNA was reduced to levels that were at or below the limit of detection (Fig. 3, lanes 5-8). When PVX-GUS was inoculated to these leaves there were as many GUS foci as on the corresponding control leaves in which there was no suppression of stGFP (Fig. 2k,l). From these differential effects on PVX-GFP and PVX-GUS we conclude that trGFP elicited sequence-specific gene silencing at the post-transcriptional level.

Example 5: The gene silencing signal is not the construct vector or host comprising the transgene coding for the fiNA

We can rule out that the systemic suppression of stGFP is associated with systemic spread of the NPT:GUS:GFP strain of *A.tumefaciens* because there was no detectable GUS[18] in tissues that exhibited systemic suppression of stGFP (Fig. 2h-j). Furthermore, using A selective enrichment procedure[19], we could not detect *A.tumefaciens* in sap extracts of tissue showing suppression of stGFP. In ten samples the selective enrichment procedure detected *A.tumefaciens* in 10^{-12}-fold dilutions of infiltrated leaf extracts. However, in forty-five samples from systemic tissue (including stems and apexes) exhibiting full or partial silencing of stGFP, the infiltrated *A.tumefaciens* was not detected, even in undiluted samples. These sensitive assay methods therefore confirm that *A.tumefaciens* cells were absent from the systemic tissue in which stGFP was suppressed. We can also rule out, based on negative results of a PCR test for GUS DNA, that there is systemic movement of the NPT:GUS:GFP binary vector independently of its *A.tumefaciens* host.

Example 6: Effect of reduced levels of fiNA

In embodiments in which the fiNA is introduced into the cytoplasm by means of transcription of a nucleic acid in the nucleus, the efficient introduction of fiNA in the cytoplasm may determine the efficiency of the silencing. To verify this the systemic silencing of GFP was only partial if the GFP constructs were modified so that the 35S promoter was either deleted or replaced with the weaker nopaline synthase promoter. The resulting partial silencing was manifest as small spots on the systemic leaves of the infiltrated plants in which there was no GFP due to stGFP. The reduced gene silencing may reflect

reduced levels of the GFP mRNA fiNA in the cytoplasm, owing to reduced transcription under a weaker promoter.

Example 7: The gene silencing signal does not require fiNA transcription

In the second series of experiments the same stGFP plants were bombarded as young seedlings with gold particles carrying DNA fragments. When the gold particles carried sequences homologous to stGFP there was silencing of GFP as described above in the infiltrated plants after 10d or more. These experiments revealed that the foreign nucleic acid need not be transcribed in order to elicit the systemic gene silencing.

CONSTRUCTS / NUCLEIC ACIDS USED FOR BOMBARDMENT:

All experiments described here involve GFP as a target gene in plants. Each bombardment is performed on 10 plants at the same time. Plants are small seedlings (usually 1cm long) grown on AGAR. The indicated nucleic acids are coated onto gold particles and the bombardment of the DNA coated gold uses electrostatic acceleration such as is well known to those skilled in the art.

Each of the following constructs / nucleic acid has been tested at least 3 times (30 plants). The ability of the construct to promote silencing is expressed in term of YIELD. The yield is calculated on the 10 bombarded plants and corresponds to number of plants showing clear systemic silencing. Silencing for these purposes was taken to mean initiation within the plant of the gene silencing signal, leading to persistent silencing of the adult plant which was essentially systemic (except in meristematic tissues and in the pollen and eggs). The systemic silencing normally becomes apparent within 10 days. post bombardment and is complete after 28 days.

1. {CamV 35S promoter - GFPcDNA - Nos terminator} in PUC19

This construct gave the most elevated yield of those tested. Out of 7 independent bombardment experiments (70 plants) the average yield of silencing is 75%.

2. {GFP cDNA} in PUC19 / pBluescript (GFP cDNA is 800 bp).

This construct gives silencing, but with an attenuated yield. It shows that transcription of the input homologous sequence (fiNA) is not required for setting the signal and the silencing throughout the plant.

Average yield calculated on 4 independent experiments (40 plants): 40%.

3. PCR-amplified fragment corresponding to the 5' part of the GFP cDNA, 400 bp long, no vector.

This gives silencing, with an average yield of 30% calculated on the basis on 3 experiments. This shows that even a portion of the target gene (here approximately the half) is able to generate silencing. Also, it shows that there is no need of a plasmid vector to carry the input sequence.

4. {3' part of the GFP cDNA, 300bp long} in PUC19

This gives silencing with an average yield of 20% calculated on the basis on 2 experiments only. This shows that (i) potentially any part of the target sequence can elicit silencing and (ii) the length and/or homology between the target and the input sequence may affect the yield of silencing, but that gene silencing can be achieved with only partial sequences.

5. Control experiments

None of the following constructs led to GFP silencing:

a. {CamV 35S promoter - GUS cDNA - Nos terminator} in PUC19 tested on 60 plants

b. {Ubiquitin promoter - GUS cDNA - Nos terminator} in PUC19 tested on 60 plants

c. {400 bp of PDS cDNA} in PUC19 tested on 40 plants

d. PUC19 tested on 30 plants

Example 8: Translocation of the gene silencing signal is facilitated by the expression of a resident gene that is homologous to the fiNA

A three-way graft was produced in which the bottom stock part was an stGFP N.benthamiana plant that had been previously infiltrated with an NPT:GUS:GFP strain of Agrobacterium as described in Example 1 and in which there was systemic silencing of GFP. The upper scion was also from an stGFP transgenic N. benthamiana but that had not been infiltrated and in which stGFP was not silenced. The intermediate scion was from a non-transgenic N.benthamiana i.e. a plant which did not comprise the GFP gene or a sequence homolog thereof. The upper part of this grafted plant remained green fluorescent over several weeks indicating that the signal did not move through the non transgenic segment that lacked a gene with homology to the fiNA. However, in Example 14 below, it was shown that after 6 weeks the signal did spread accross the graft junction in a number of cases, indicating that transcription of a homologous gene is not an absolute requirement for transmission.

In separate experiments it was confirmed that the signal of gene silencing did move efficiently though the graft union between the stock and scion of two stGFP plants.

Example 9: TIGS is stably maintained whereas VIGS is not

stGFP N. benthamiana plants were infected with PVX-GFP to elicit 'viral induced gene silencing' ('VIGS') of GFP or were infiltrated with an NPT:GUS:GFP strain of Agrobacterium to induce TIGS. The VIGS had extended through most of the upper part of the plant by 21 days post inoculation and associated with this there was suppression of PVX-GFP below the levels detectable northern blotting. By 35 days the uppermost regions of the plants regained green fluorescence indicating that VIGS had diminished although there was no reappearance of the PVX-GFP. This suggests that VIGS requires continued presence of the initiator virus.

In the plants exhibiting TIGS of GFP the initial spread of gene silencing was at the same rate as in the plants showing VIGS. However, in these plants the silenced condition was permanent for 42 days or longer after the initial infiltration. All upper parts of the plant except the meristems, pollen and eggs exhibited silencing of GFP. The silenced condition remained even if the infiltrated leaf was detached. Thus TIGS does not require continued presence of the fiNA.

Example 10 - The TIGS can be maintained in regenerated plants

It was even possible to regenerate stGFP silenced plants by tissue culture of leaf disc explants from the upper parts of the TIGS plants. These regenerated plants showed silencing of stGFP in the same way as the original infiltrated plants.

The regeneration of gene silencing plants may be carried out by methods analogous to those used by those skilled in the art for regeneration of plants. Briefly, the regeneration was carried out as follows:

1) take a leave from a silenced plant (silenced by TIGS)
2) sterilize it for 30 minutes in 7.5% domestos
3) cut the leaf into small squares
4) put this square into "MS media plus vitamins" (Sigma) supplemented with 1.0 mg/ml of 6-BAP, 0.1 mg/ml of NAA, 3% sucrose.
5) after 2-3 weeks the squares start to produce shoots that are completely silenced (except on meristems).
6) transfer these shoots to unsupplemented "MS media plus vitamins"
7) allow the plants to grow

The post transcriptional silencing was evidenced by a continued resistance to viral constructs sharing homology with the silenced gene, but no resistance to other viral constructs which did not include a GFP sequence or homolog thereof.

Example 11 - The TIGS signal has the characteristics of nucleic acid

GFP transgenic N.benthamiana were harvested at 10-20 d post infiltration with the NPT:GUS:GFP strain of agrobacterium and the leaves in which GFP expression was silenced were homogenised in phosphate buffer (50 mM pH7.0). The homogenate was then applied to the leaves of GFP N.benthamiana that had not previously been infiltrated and in which GFP expression was not silenced. The procedure for application of the sap was the same as standard procedures used to inoculate plants with virus-infected sap: the leaves were first dusted with carborundum. A drop of sap (20uL) was applied to the

leaves and the leaves were rubbed gently by hand to generate abrasions through which the sap components could enter the cells. After five minutes the leaves were drenched with water so that residual sap would not have a toxic effect.

By 20 days post treatment the GFP expression was largely unaffected. However there were several (5-20) small regions on each plant in which GFP expression (diagnosed by absence of green fluorescence under UV light) was absent. These regions varied in size between 1 and 10mm diameter. There were no regions of GFP suppression if the extracts were taken from GFP N.benthamiana that had not previously been infiltrated with the NPT:GUS:GFP strain of agrobacterium or from non transgenic plants.

The presence of the regions suppressed GFP expression indicates that the signal of silencing had been isolated in the sap extracts. We conclude that this signal is a nucleic acid because it was heat labile (100°C 5 min) and was not destroyed when the sap was extracted with phenol/chloroform. The signal was also not destroyed by DNAase treatment of the sap indicating that it may be RNA.

Example 12: TIGS is not the same as VIGS

stGFP N.benthamiana were inoculated with a mutant derivatives of PVX-GFP in which the CP gene had been deleted. Because of this mutation the virus was disabled for cell to cell movement. Whereas the intact PVX-GFP elicited systemic silencing of the GFP transgene in a manner consistent with the systemic spread of the virus throughout the plants, these mutant constructs failed to do so. This failure was not because the inocula were inactive: the same inocula applied to transgenic plants expressing the PVX CP produced croning infection loci due

to complementation of the CP mutation in the virus.

This result shows that VIGS did not produce a signal that moved long distances beyond the infected cells: the systemic effect of VIGS must be because the virus can move between cells. In contrast, TIGS, despite the involvement of a fiNA that is not endowed with cell to cell movement properties, does produce a long distance signal as described in the above examples.

In Examples 13 to 19 below, the stably integrated GFP transgene (trGFP) is referred to as "intGFP", while the transient FINA GFP (trGFP) is referred to as "epiGFP".

Example 13: The gene silencing signal requires uptake of the transgene coding for the fiNA : The role of T-DNA transfer and transcription

As discussed in Example 3 above, transfer of the T-DNA from A. tumefaciens to the plant cell nucleus is a process that requires expression of the bacterial virulence (Vir) genes. To determine whether TIGS requires transfer of epiGFP into plant cells, the previously described experiments were repeated under conditions in which the A. tumefaciens Vir gene activity was either up- or down-regulated. To down-regulate the Vir genes, the A. tumefaciens culture was incubated prior to infiltration in the absence of acetosyringone, which is an inducer of Vir genes (Ream, 1989). Up-regulation of Vir genes was achieved by use of a hypervirulent strain of A. tumefaciens (cor308) carrying duplicate copies of VirG, VirE1 and VirE2 (Hamilton et al., 1996). VirG is the transcription activator of all Vir functions; VirE1 and VirE2 are involved in T-DNA transfer and stabilisation in the cytoplasm. VirE2 is also required for nuclear targeting of the T-DNA (Zupan and Zambryski, 1997).

Both approaches indicated that TIGS requires Vir gene function. Thus, with N:G:G, A. tumefaciens cultures produced in the absence of acetosyringone, the onset of TIGS was inconsistent from plant to plant and was much slower (40d post infiltration) than with cultures prepared in the presence of acetosyringone (around 20d post infiltration) as shown in Table I:

Table 1. Effect of *A. tumefaciens Vir* genes and epiGFP promoters on TIGS.

Binary vector	aceto-syringone induction	hyper-virulent strain cor308	No. of plants	No. silenced plants by 7 dpi	No. silenced plants by 20 dpi
N:G:G	+	+	30	26	30
N:G:G	+	-	100	0	100
N:G:G	-	-	30	0	0
N:G	+	-	30	0	30
N:Gnos	+	-	30	0	30
N:G Δ	+	-	30	0	30

"dpi" is an abbreviation for d post infiltration. A plant was considered as silenced if there was loss of GFP fluorescence surrounding the veins of systemic leaves.

Furthermore, when cultures were produced without acetosyringone, TIGS was restricted to small discrete zones in the upper parts of the infiltrated plants and was much less extensive than in plants infiltrated with acetosyringone-treated cultures. Conversely, the use of a hypervirulent A. tumefaciens (cor308) host of the N:G:G construct accelerated the development of TIGS by several days: TIGS initiated with this strain started at 7d post infiltration and was complete by 10d (Table I).

The influence of Vir gene expression indicates that TIGS requires transfer of T-DNA into plant cells. However, these experiments do not show whether epiGFP transcription is required. To address this issue, the infiltration experiments were repeated with derivatives of the pBin35S:GFP construct (Figure 1) in which the 35S promoter of epiGFP was either replaced with the nos promoter (N:Gnos, Figure 4). The nos promoter is much weaker than the 35S promoter of CaMV (Harpster et al., 1988). We also agroinfiltrated with a construct without a GFP promoter (N:G Δ, Figure 4). In several experiments (Table I) there was TIGS of intGFP when the constructs were infiltrated into transgenic N. benthamiana plants. With both of these constructs, TIGS developed as quickly as with the original N:G:G construct (Table I), indicating that the presence of a promoter upstream epiGFP is not required for initiation of TIGS.

Example 14 - Propagation of the TIGS signal

Symplastic movement of molecules in plants can occur from cell-to-cell through plasmodesmata and/or through the phloem (Lucas et al., 1989). To investigate which of these routes is used to propagate TIGS, we monitored intGFP silencing after infiltration of plants with the N:G:G strain of A. tumefaciens. At 20d post-infiltration of lower leaves, the silencing was manifest in systemic, young developing leaves and was very pronounced in the shoot tips. There was also silencing in upper leaves that were already expanded at the time of infiltration but it was fainter and less extensive than in the young developing leaves. In contrast, the leaves immediately above and below the infiltrated leaves remained fully green fluorescent. At 30d post-infiltration the stem and roots below the infiltrated leaves also showed intGFP silencing, thus indicating that the movement of the TIGS signal was bi-directional in the plant. In terms of speed

and spatial distribution, this pattern of spread is similar to the movement of viruses in the phloem, from source to sink leaves (Leisner and Turgeon, 1993).

Additional support for phloem transport of the signal comes from experiments in which intGFP plants were infiltrated with the N:G:G strain of A. tumefaciens in just a single leaf. These experiments differ from those described previously in which the plants were infiltrated in two or three leaves on opposite sides of the plant. At 1 month post-infiltration, intGFP silencing in the stem was restricted to the side of the original infiltrated leaf. Shoots that had emerged from the silenced portion of the stem were silenced, while those emerging from the non-silenced half were not. This pattern of signal movement was strikingly similar to the spread of a phloem-translocated dye and of a systemic virus in N. benthamiana (Roberts et al., 1997).

The development of silencing in leaves was also similar to the translocation of a phloem-transported dye through class I, II and III veins of N. benthamiana leaves (Roberts et al., 1997). In systemic leaves that had already expanded at the time of infiltration, intGFP silencing was initially (20d post infiltration) in regions surrounding the main veins and later (27d post-infiltration) in regions around the minor veins. At 34d post-infiltration, intGFP silencing spread across the whole lamina of the leaf thus indicating that there was cell-to-cell movement of the silencing signal as well as translocation through the phloem. This cell-to-cell movement is likely to occur through plasmodesmata because there was no intGFP silencing in the stomatal guard cells which would have been symplastically isolated before the signal moved into the leaf (Ding et al., 1997; McLean et al., 1997). However, in leaves that developed after the signal had spread to the apical growing point, intGFP was

uniformly silenced, even in the stomatal guard cells. From this observation, we conclude that guard cells are competent for gene silencing provided that the signal invades leaves early in their development, before symplastic isolation of the guard cells.

To further investigate the movement of the TIGS signal, we carried out grafting experiments similar to those described previously to characterise systemic spread of transgene-induced gene silencing (Palauqui et al., 1997; see also Example 8 above). Specifically, we wished to determine whether the signal could move through cells in which there were no genes with sequence similarity to the target of TIGS. First, to confirm that the signal is graft transmissible, we wedge-grafted non-silenced intGFP scions onto intGFP rootstocks exhibiting TIGS. TIGS spread into the scions about four weeks after the graft union in 10 out of 16 graftings tested. As with the intact N:G:G infiltrated plants, intGFP suppression in the scions was first manifest around the veins of newly emerging leaves and later became widespread on all vegetative parts of the scions.

Having thus established that the signal in this system is graft transmissible, we produced three-way grafts comprising a silenced intGFP rootstocks, an intermediate section of nt stem and a top scion of a non silenced intGFP plant. Using this procedure, we observed silencing occurring in the intGFP top scions about six weeks after the graft junctions in 5 out of 11 graftings tested. This result demonstrates that the TIGS signal could move long distances and through cells in which there is no corresponding nuclear gene, as the intermediate section had no GFP sequence.

In a separate series of experiments, the speed of signal movement was assessed by removal of the infiltrated leaf

1, 2, 3, 4 or 5 days after infiltration with the N:G:G strain of A. tumefaciens. In these experiments, there was systemic loss of intGFP fluorescence (i.e. TIGS) in 10% of the plants if the infiltrated leaf was removed 2d post-infiltration. A progressively higher proportion of plants exhibited TIGS when the infiltrated leaf was removed 3d or later (Figure 5). From these data, we conclude that production and translocation of the signal occurs within 2 or 3d post-infiltration.

In plants that exhibited TIGS after removal of the infiltrated leaf, loss of intGFP developed as quickly and persisted for as long as in the intact plants. Furthermore, in all of the N:G:G-infiltrated plants, TIGS of intGFP persisted for more than 100d post infiltration. Even in these old plants, TIGS continued to be induced in the newly emerging leaves, despite the loss of the infiltrated leaf due to senescence. Considering these observations, we propose that propagation of the TIGS signal occurs via a relay process. The cells receiving the signal from the infiltrated leaf would become a secondary source of the signal so that maintenance of PTGS in the plant would become independent of the infiltrated leaf.

Example 15 - TIGS in meristematic cells

Although there was extensive and persistent silencing of intGFP in the N:G:G-infiltrated N. benthamiana plants the floral, vegetative and root apexes always remained non silenced i.e. green fluorescent (see below). Either the signal of gene silencing cannot enter dividing cells or dividing cells lack the potential to silence intGFP. To address these alternatives, we cultured leaf explants from plants exhibiting TIGS of GFP. The explants were cultured on media promoting shoot regeneration. It was expected that intGFP silencing would be lost if dividing

cells lack the potential to silence intGFP.

In shoots and leaves regenerating from these explants there was no intGFP fluorescence in most parts of the organs, whereas shoots regenerated from non-silenced plants remained fully green fluorescent. From these observations we conclude that silencing was not induced by the culture procedures but that it could persist through in vitro organogenesis. However the extreme apical regions of the silenced shoots were green fluorescent, as in the progenitor plants. When the shoots developed into plants with roots, the root tips and apical zones of vegetative and floral shoots were also green fluorescent. This apical fluorescence was not present in nontransformed plants and is therefore bona fide GFP rather than an artefact due to the presence of fluorescent compounds. These results indicate that TIGS can be maintained in, or can pass through dividing cells but that the gene silencing mechanism is not effective in meristematic tissues of the plant, presumably because the signal of TIGS cannot reach those regions. These findings reinforce the striking similarities between the movement of the TIGS signal and the movement of plant viruses, which are generally excluded from meristems (Matthews, 1991).

Example 16 - Biolistic activation of TIGS

In the experiments described above, epiGFP was delivered by infiltration of A. tumefaciens into leaves of intGFP transgenic plants. To evaluate an alternative means of epiGFP delivery, we bombarded small seedlings (5-7 mm long) with gold particles coated with the pUC 35S-GFP plasmid (Figure 6A). This plasmid is based on pUC19 and has the complete 35S-GFP cassette from pBin35S-GFP (Figure 6A). Three weeks after bombardment, 75% of the plants showed TIGS of intGFP. As in the agroinfiltrated

plants, there was TIGS of intGFP throughout the plant except in the growing points of the shoots and roots. This result was consistent and reproducible in seven independent experiments, involving a total of 70 plants (Figure 6A). TIGS of intGFP was never observed when intGFP plants were bombarded with uncoated gold particles or plasmid that did not carry the GFP ORF (data not shown). In order to estimate the number of cells that receive the delivered DNA, we also bombarded seedlings with a pUC 35S-GUS plasmid and stained the whole plants for GUS activity three days later. We found that, on average, less than 8 randomly distributed individual cells exhibited blue staining in whole seedlings. These results indicate that TIGS does not depend on the delivery method of epiGFP and that very localised events can initiate production and spread of the sequence-specific signal of gene silencing.

Bombardment of linear fragments of GFP cDNA without a promoter, either intact or as 5′ or 3′ fragments, also led to TIGS. The two fragments of GFP (..P and G..; Figure 6A) were both less efficient initiators of TIGS than the intact cDNA (GFP, Figure 6A) thus indicating that initiation of TIGS is affected by the length of epiGFP. To further investigate importance of epiGFP length, a series of PCR-amplified fragments were produced. These fragments were all of the same physical length (500bp) but had 3′ co-terminal fragments of GFP cDNA of varying length. The non-GFP DNA in these fragments was from pBluescript. Equal amounts of each fragment were bombarded into 50 plants in 5 independent experiments. The results, summarised in Figure 6B, clearly show that the efficiency of TIGS initiation is determined by the length of homology between the epiGFP and the intGFP.

Example 17: TIGS requires an interaction of epiGFP and

intGFP

In principle, TIGS could be initiated by epiGFP alone. Alternatively it could be initiated following an interaction between epiGFP and intGFP DNA or intGFP RNA. To distinguish between these possibilities, we have further characterised the targets of TIGS following bombardments with 5′ or 3′ linear fragments of GFP cDNA (GF. and..P, Figure 7A). If TIGS was initiated only by the bombarded DNA, the target would be confined to the region (i.e. sequence) of the bombarded DNA. However, a target that was determined following an interaction with intGFP could extend beyond the regions of the bombarded DNA. The assay for TIGS target sites involved inoculation of PVX-GF and PVX-P (Figure 7A) to intGFP plants that had been bombarded 21d previously with GFP, ..P or GF. (Figure 7A, diagram). Virus inoculations were made to leaves exhibiting TIGS of intGFP and accumulation of the viral RNA was assessed by northern analysis of RNA samples taken from the inoculated leaves at 8d post inoculation (Figure 7A, diagram).

Northern analyses of inoculated leaves showed that accumulation of PVX-GFP and PVX-GF (Figure 7B, lanes 8-10 and 12-14) was lower (by at least ten fold) in leaves exhibiting TIGS of intGFP than in the leaves of non transformed plants (Figure 7B lanes 6) or in the leaves of intGFP plants that had been previously bombarded with uncoated gold particles (Figure 7B, lanes 6,7 and 11). It was particularly striking that silencing induced by epi..P could target PVX-GF (Figure 7B, lanes 13 and 14) and, conversely, silencing induced by epiGF. could target PVX-P (Figure 7A, data not shown). As there is no sequence overlap between the GF. and ..P fragments involved in these experiments, we conclude that the target site of TIGS is determined following an interaction of epiGFP and intGFP DNA or intGFP RNA.

Moreover, the influence of the bombarded DNA can extend both in the 3' (from GF to P) or in the 5' (from P to GF) direction.

Example 18 - Spontaneous TIGS

Among our transgenic N. benthamiana lines, we identified one line (15a) in which intGFP systemic silencing occurs spontaneously. As with many examples of PTGS in plants, the silencing phenotype of line 15a is influenced by transgene dosage (Hobbs et al., 1993)(Mueller et al., 1995). Progeny of 15a with a hemizygous GFP transgene remained green fluorescent (data not shown) whereas those with a homozygous transgene exhibited intGFP silencing. The development of silencing in these plants followed the same pattern as in infiltrated and bombarded plants. Initially, the plants were uniformly green fluorescent but, at the four leaf stage, spots of red fluorescence developed around the veins of the upper leaves. Eventually, these regions spread along the length of the veins and throughout the plant as for TIGS induced by bombardment or infiltration of A. tumefaciens. We confirmed by grafting experiments the involvement of a systemic signal of silencing in line 15a. In addition, intGFP silencing was not observed in 15a meristems, as in plants exhibiting TIGS. From these observations we conclude that the bombardment or A. tumefaciens infiltration mimic processes that can take place spontaneously in transgenic plants.

Example 19 - TIGS from viral constructs - effect of viral proteins

A number of constucts were prepared based on the PVX-GFP amplicon constructs of PCT/GB98/00442, but included various deletions in the PVX or transgene regions. GFP was monitored under UV light.

Construction of plasmids

Referring to Figures 8 to 10.

The constructs were based upon pPVX209 (in which PVX-GFP is inserted into a pUC19 plasmid under a 35S promoter) which in turn was based on pPVX204 (see Baulcombe et al, 1995) but including an additional SacI site at the 5' side of the promoter.

Plasmid pPVX210A, which included a coat protein (CP) deletion, was generated from pPVX209.

Plasmids pCL100, pCL101 and pCL102, which included further deletions in the 'triple block' of cell-to-cell movement proteins (25K, 12K and 8K), were generated from pPVX210A.

Plasmid pCL105, which included further deletions in the replicase (Rep) region, was generated from pCL100.

Plasmid pCL106 included a PCR fragment from pPVX210A to restore GFP function.

Fig 10 shows how the pUC19 constructs were inserted into the Agrobacterium binary vector plasmid pSLJ755/5. These constructs are numbered as per Table 2:

Description of construct	Construct in pUC19	Construct in pSLJ755/5
PVX-GFP-CP	pPVX209	pPVX211
PVX-GFP	pPVX210A	pPVX212A
PVX-ΔB-FP	pCL102	pCL112
PVX-ΔGV-FP	pCL101	pCL111
PVX-ΔTGB-FP	pCL100	pCL110

PVX-ΔRepΔTGB-FP	pCL105	pCL115
PVX-ΔTGB-GFP	pCL106	pCL116

PVX-GUS	pA500	-
PVX-ΔB-GUS	pCL104	pCL114
PVX-ΔTGB-GUS	pCL103	pCL113

Table. 2. List and description of minimal constructs created (in bold type), and progenitor constructs.
ΔB: TGB deletion retaining 5′ UTR of TGB and 5′ end of 25-kDa protein gene
ΔGB: TGB deletion retaining the 5′ UTR of TGB
ΔTGB: TGB deletion retaining only the first 3 nt. of the UTR of TGB

The positive strand sequences for some of the constructs are given in Fig 11.

Production and Replication of viral RNA in infected cells

This was confirmed in wild-type plants. Owing to the fact that movement proteins were disabled in most constructs, a standard infection assay could not be used. However, Agrobacterium strains could be infiltrated into the leaves of *N. benthamiana* to infect a high density of cells in a region of the infiltrated leaf. Northern analysis of RNA isolated from the infiltrated zone of the leaf showed that there was replication of the transcripts from constructs 212A, 110, 112 and 116 as would be predicted from their structure. The 116 construct, which included the strong CP sub-genomic promoter, produced more subgenomic RNA than other constructs. Similarly, under UV light the 212A and 116 gave bright green fluorescence - brighter than a 35S-GFP construct (pA1036 - not shown) which is again consistent with

replication of the constructs.

Use of constructs to generate TIGS

Silencing of a GFP-transgenic plant was assessed as described in earlier examples in relation to non-replicating 35S-GFP constructs. The constructs described above were introduced into Agrobacterium tumefaciens (strain GV3101) and cultures were allowed to grow in the presence of acetosyringone. The leaves of a GFP transgenic plant were then infiltrated with the agrobacterium, as described in Example 1, and gene silencing was monitored over a four week period by UV illumination of the plants. The PVX-GFP construct in pPVX212A (see Table 2) was a less efficient silencer sequence than the PVX-Drep-DTGB-FP construct whereas the PVX-DTGB-FP (pCL110) and PVX-DTGB-GFP (PCL116) were more efficient than PVX-Drep-DTGB-FP. From these data we conclude that the ability to produce a replicating RNA, although not necessary to perform the invention, greatly enhances the efficiency of silencing but that the viral movement proteins (encoded in pPVK212A but not in PVX-DTGB-FP (pCL110) and PVX-DTGB-GFP (PCL116)) are antagonists of gene silencing. We conclude that constructs for gene silencing should be constructed so as to avoid expression of movement proteins that may antagonise the gene silencing mechanism.

DISCUSSION OF EXAMPLES 13-19

These Examples employ TIGS to further dissect PTGS into separate initiation, spread and maintenance stages. In this discussion we assess the likely molecular mechanisms of these different stages and the natural role of gene silencing in plants and other organisms. We consider the spread stage first, because the inferences about the likely nature of the signal of gene silencing influence

the subsequent discussion about the initiation and maintenance stages of gene silencing.

Systemic spread of TIGS

Systemic spread of TIGS is remarkable in that it involves a sequence specific signal: TIGS initiated against GFP was specific for intGFP or viral GFP RNAs whereas TIGS against GUS was specific for GUS RNAs. This pattern of sequence specificity rules out the possibility that TIGS is a non specific wounding signal or that the specificity is related to the 35S promoter. Therefore it is likely that the signal of TIGS is specific for the transcribed regions of the target gene and that the specificity determinant includes a nucleic acid component. Thus, the signal for TIGS of GFP is likely to contain GFP RNA or DNA, whereas the signal for TIGS of GUS or other genes would contain the corresponding alternative nucleic acid species. From its pattern and speed of systemic spread, we confirm that this putative nucleic acid is able to move not only from cell to cell through plasmodesmata but also systemically through the phloem, as proposed in a recent review article (Jorgensen et al., 1998).

There are precedents in plants for endogenous nucleic acids that move between cells. For example, there are mobile nucleic acids encoded by nuclear genes including the mRNA for a transcription factor (Lucas et al., 1995) and a sucrose transporter mRNA (Kuhn et al., 1997). However in both of these examples the movement is only between cells: there is no evidence for long distance movement, as with the signal of TIGS. The mobile nucleic acids that are most obviously comparable to the putative signal of gene silencing are viroids. Like the signal of silencing (Figure 5), these small non-coding RNA species move systemically within a period of a few days after inoculation (Palukaitis, 1987). For both viroids and

TIGS, the route of movement involves cell-to-cell through plasmodesmata and long distance spread through the phloem (Palukaitis, 1987; Ding et al., 1997).

From the leaf detachment experiment (Figure 5), we infer that movement of the signal involves a relay. Some cells receiving the epiGFP were the primary source of initial signal production. However, once the signal moved out of the bombarded or infiltrated leaves this primary source was no longer required and there must have been cells elsewhere in the plant that were a secondary source of the signal molecule. We do not know the maximum distance between primary and secondary relay points in signal production but, from the three-way grafting experiments, we can infer that distances of several centimetres or more could be involved.

Also of interest is the deduced effect of the viral movement proteins on the spread (or possibly the initiation) of the signal (Example 19). This suggests that, while it may be desirable to have replicating constructs as a source of the fiNA, it may also be desirable to limit these to only a replicase, plus associated cis acting elements and targeting sequence, all under the control of a suitable plant promoter.

Initiation and maintenance of signal production

TIGS was initiated in the bombarded or infiltrated cells that received epiGFP. It is unlikely, although it cannot formally be ruled out, that TIGS required transcription of the introduced DNA because the presence of a promoter had little or no effect on the initiation of TIGS (Table I above, plus also Figures 6 and 7). It is also unlikely that the signal was derived directly from the introduced DNA because TIGS induced by ..P resulted in targeting of the GF. component of GFP RNA. Similarly, bombardment of

GF. produced silencing targeted against ..P (Figure 7). Our interpretation of these data is that TIGS was initiated by an interaction between intGFP and epiGFP and that the target of TIGS was determined by intGFP. The influence of epiGFP length on TIGS is also consistent with an homology-dependent interaction between epiGFP and intGFP (Figure 6B).

We recognise that this proposed interaction of epiGFP could involve intGFP DNA or RNA and that our data do not provide conclusive evidence for either. However, we consider that an interaction with DNA is more likely than with RNA because in N:G:G and N:G_{Δ} the GFP transgene was orientated 5′ to 3′ towards the left border of the T-DNA (Figure 4B). The orientation of this gene is relevant because the T-DNA of A. tumefaciens is transferred into plant cells as single-stranded DNA with the right border of the T-DNA at the 5′ end (Zupan and Zambryski, 1997). This strand-specific transfer mechanism would not allow the single stranded epiGFP DNA to interact with intGFP RNA because both molecules have the same polarity. However, the single-stranded epiGFP T-DNA would have the potential to interact with homologous DNA in the genome, irrespective of the orientation of the insert. Consistent with a DNA-level interaction we have also shown that single stranded GFP DNA with the polarity of intGFP RNA can initiate TIGS after bombardment (data not shown).

How could a DNA-level interaction of epiGFP and intGFP result in TIGS ? We propose here a mechanism similar to an earlier ectopic pairing model of PTGS in transgenic plants. According to this model, the ectopic interactions of epiGFP and intGFP would perturb transcription of the intGFP and lead ultimately to formation of anti-sense RNA (Baulcombe and English, 1996). This antisense RNA would target GFP RNAs for degradation and would be a component of the signal molecule. If the DNA-level interaction led

to aberrant transcription of the non-coding strand of the genomic DNA, this antisense RNA could be a product of direct transcription from the genome. Alternatively the anti-sense RNA could be produced indirectly by a host-encoded RNA-dependent RNA polymerase, as suggested originally to explain transgene mediated PTGS (Lindbo et al., 1993). In this scenario the RNA-dependent RNA polymerase would produce anti-sense RNA using aberrant sense RNA as template.

The proposal that there could be ectopic interactions of homologous DNA leading to aberrant transcription is based on precedents from plants, animals and fungi. In one example, with β-globin genes in mammalian cells, an ectopic DNA interaction was demonstrated directly by the co-localisation of a transfected plasmid with the homologous sequence in the genome (Ashe et al., 1997). In plant and fungal cells, the ectopic interaction could only be inferred indirectly from the modified methylation pattern of the homologous DNAs (Hobbs et al., 1990; Barry et al., 1993). We envisage that these ectopic interactions may lead to aberrant RNA either by arrest of transcription leading to prematurely truncated RNA species, as shown in Ascobolus immersus (Barry et al., 1993). Alternatively the ectopic interactions could cause aberrant extension of transcription, as in the example with β-globin genes (Ashe et al., 1997).

A DNA-level interaction leading to aberrant transcription provides a convenient explanation for the persistence and uniformity of TIGS in the plant. For example, it would explain why the silenced state was stable during the lifetime of the silenced plant. The interaction of the introduced DNA or the signalling molecule at the DNA level could lead to an epigenetic change involving DNA methylation or chromatin modification that could persist even if the silenced cell was no longer receiving signal.

Consistent with this hypothesis, it has been shown that viroid RNAs can direct sequence-specific DNA methylation in transgenic plants (Wassenegger et al., 1994). Furthermore, transcription of the epimutated DNA or chromatin could provide an amplification step in TIGS. This amplification would explain the relay of TIGS and why the signal does not get diluted as it moves away from the initially infiltrated or bombarded cells.

TIGS compared to other examples of gene silencing in plants and animals.

Many examples of gene silencing in plants may be similar to TIGS. For example, in transgenic plants exhibiting transgene-induced PTGS, it is clear from grafting experiments (Palauqui et al., 1997) and from the spatial patterns of silencing that there is an extra-cellular signal of silencing. In addition we consider it likely that gene silencing with a delayed onset, for example with GUS transgenes, may also involve systemic spread of a signal (Elmayan and Vaucheret, 1996). In these instances, we envisage that the process may be initiated in just one or a few cells in the plant, as shown here in TIGS, and that the spread of the signal accounts for the gene silencing throughout the plant.

The involvement of a signal molecule means that genetic or epigenetic variations in single cells could influence the level of gene silencing throughout the plant. Consequently, the analysis of transgenes in whole plant DNA may not be an accurate indicator of factors that influence PTGS. For example, in a previous study based on analysis of whole plant DNA, it was concluded that single copy, hemizygous transgenes can activate PTGS (Elmayan and Vaucheret, 1996). This conclusion was difficult to reconcile with the suggestion that ectopic DNA interactions initiate PTGS (Baulcombe and English, 1996).

However, the results presented here show that the PTGS in the whole plant could have been initiated in individual cells carrying multiple copies of the transgene due to DNA endoreduplication or chromosomal rearrangements. Therefore, even in plants having only one copy of a silencer transgene in the genome, it cannot be ruled out that PTGS was initiated by ectopic interactions of homologous DNA.

Most analyses of PTGS have involved plants and fungi. However there are now reports of gene silencing phenomena in animals that appear similar to the plant and fungal systems. For example, in Drosophila melanogaster there is co-suppression of transgenes and endogenous genes as in petunia, tobacco and other plant systems (PalBhadra et al., 1997). However, more striking, are two recent examples of gene silencing in Caenorhabditis elegans (Fire et al., 1998) and in Paramecium (Ruiz et al., 1998a). The "genetic interference" described in C. elegans is initiated by double stranded RNA (Fire et al., 1998) rather than DNA, as described here, but otherwise shares many common features with TIGS including the ability to spread by a relay mechanism through the affected organism. In Paramecium, microinjection of plasmids containing sequences of a gene led to homology-dependent silencing of the corresponding gene in the somatic macronucleus (Ruiz et al., 1998a). As described here for TIGS, the silencing effect could be initiated with plasmids containing only the coding region of the gene and was stably maintained throughout vegetative growth of the organism. Perhaps the similarity between TIGS, the induced silencing in Paramecium and the effect of double stranded RNA in C. elegans reflects the existence of a ubiquitous mechanism in plants and animals that is able to specifically target aberrant RNA. This possibility fits well with the suggestion that RNA double-strandedness is a possible aberrance required for

initiation of PTGS in transgenic plants (Metzlaff et al., 1997).

A role for TIGS in plants?

In addition to the previously made suggestion that TIGS reflects a protection mechanism in plants against viruses and transposons (Voinnet and Baulcombe, 1997 - see also above), we consider it possible that TIGS also represents a natural signalling mechanism in plant development. These proposals were anticipated in an insightful review written in 1982 suggesting that viroids exploit a natural mechanism of RNA signalling (Zimmern, 1982). We consider it is possible, for example, that TIGS-like signalling may be implicated in the control of flowering in plants. It is known from classical experiments that there is a graft transmissible signal of flowering (florigen) which has many of the predicted attributes of a natural manifestation of TIGS (Poethig, 1990). Like the TIGS signal, florigen does not correspond to any of the conventionally characterised hormones or other signalling molecules in plants but it does move systemically to produce an epigenetic switch (Bernier, 1988; Colasanti et al., 1998). With florigen the epigenetic switch is associated with the transition from the vegetative to the flowering state of the plants and in TIGS, gene silencing can be considered as an epigenetic event. In some instances changes in DNA methylation have been implicated in floral commitment (Poethig, 1990). Perhaps florigen and the putative signal of TIGS are similar types of mobile RNA. This RNA might have the characteristics of viroid RNA that allow it to move systemically in plants and direct sequence specific DNA methylation (Wassenegger et al., 1994). In the case of florigen the target DNA might be sequences controlling the transition from the vegetative to the flowering state.

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CLAIMS

1. A method for silencing a target nucleotide sequence present in a first part of a plant, which method comprises transiently introducing into the cytoplasm of a cell in a second part of the plant, which cell comprises a nucleic acid encoding the target sequence and which is remote from said first part of the plant, a nucleic acid construct, wherein said construct:

(i) encodes a sequence which shares sequence identity with the target nucleotide sequence or the complement thereof, and

(ii) does not encode proteins which are capable of blocking systemic movement of a gene silencing signal,

such that a silencing signal not comprising the construct is initiated in the first part of the plant and propagated to the second part of the plant such as to cause the silencing of said target nucleotide sequence.

2. A method as claimed in claim 1 wherein the proteins which are capable of blocking systemic movement of a gene silencing signal are those which are capable of mediating intercellular viral movement.

3. A method as claimed in claim 1 or claim 2 wherein the part of the plant into which the nucleic acid is introduced corresponds to a region in which photosynthetic products are concentrated and the target nucleotide sequence is present in a remote region in which such products are used.

4. A method as claimed in any one of the preceding claims wherein the target nucleotide sequence, or a nucleotide sequence sharing homology with the target nucleotide sequence, is transcribed in the cells of the tissues connecting the first and second parts of the plant through which the gene silencing signal is

propagated.

5. A method as claimed in any one of the preceding claims wherein the target nucleotide sequence is silenced systemically in the plant.

6. A method as claimed in any one of the preceding claims wherein the construct is not capable of autonomous replication.

7. A method as claimed in any one of the preceding claims wherein the construct introduced into the plant cell does not encode a viral coat protein

8. A method as claimed in any one of the preceding claims wherein the sequence sharing sequence identity with the target gene does not include translation-recognition signals such that said sequence is not translated to a protein product.

9. A method as claimed in any one of the preceding claims wherein the nucleic acid construct is DNA.

10. A method as claimed in any one of the preceding claims wherein the construct comprises a promoter operably linked to a nucleotide sequence, wherein said nucleotide sequence:
(i) encodes a viral replicase,
(ii) encodes a replicable sequence which shares sequence identity with the target nucleotide sequence or its complement, and which is operably linked to one or more cis acting elements recognised by said replicase, such that the replicable sequence is replicated in the cytoplasm of the cell into which it is introduced,
(iii) does not encode proteins which are capable of mediating intercellular viral movement.

11. A method as claimed in claim 11 wherein the viral replicase is a PVX replicase.

12. A method as claimed in claim 10 or 11 wherein the promoter is an inducible promoter.

13. A method as claimed in any one of claims 10 to 12 wherein the construct comprises Ti-derived sequences which permit integration of the construct into the plant genome.

14. A method as claimed in any one of claims 1 to 9 wherein the construct does not comprise any of the following:
(i) promoter or terminator sequences,
(ii) Ti-derived sequences which permit integration of the construct into the plant genome.

15. A method as claimed in claim 13 wherein the construct is introduced into the plant using Agrobacteriun tumafaciens.

16. A method as claimed in any one of claims 1 to 14 wherein the construct is introduced into the plant cell by microprojectile bombardment.

17. A method as claimed in any one of the preceding claims wherein the target nucleotide sequence encodes a heterologous gene.

18. A method as claimed in any one of claims 1 to 16 wherein the target nucleotide sequence encodes a gene which is endogenous to the plant.

19. A method as claimed in claim 18 wherein the plant is not a transgenic plant.

20. A method as claimed in any one of the preceding claims wherein the target nucleotide sequence encodes all or part of a viral genome of a virus in the plant.

21. A method as claimed in any one of the preceding claims wherein two or more target genes which share sequence identity are silenced.

22. A method of assessing a phenotypic characteristic associated with a target nucleotide sequence in a plant, the method comprising:
(a) silencing the nucleotide sequence in a plant in accordance with a method as claimed in any one of the preceding claims,
(b) observing the phenotype of the plant, and optionally
(c) comparing the result of the observation with the phenotype of a control plant.

23. A method for regulating the expression of a target nucleotide sequence in a plant comprising use of a method as claimed in any one of claims 1 to 21.

24. A method of systemically altering the phenotype of a plant comprising use of a method as claimed in any one of claims 1 to 21.

25. A nucleic acid construct comprising a promoter operably linked to a nucleotide sequence, wherein said nucleotide sequence:
(i) encodes a viral replicase,
(ii) encodes a replicable sequence which shares sequence identity with the target nucleotide sequence or its complement, and which is operably linked to one or more cis acting elements recognised by said replicase, such that the replicable sequence is replicated in the cytoplasm of the cell into which it is introduced,
(iii) does not encode proteins which are capable of

mediating intercellular viral movement.

26. A construct as claimed in claim 25 which is a DNA plasmid.

27. A construct as claimed in claim 26 which is a Ti plasmid vector.

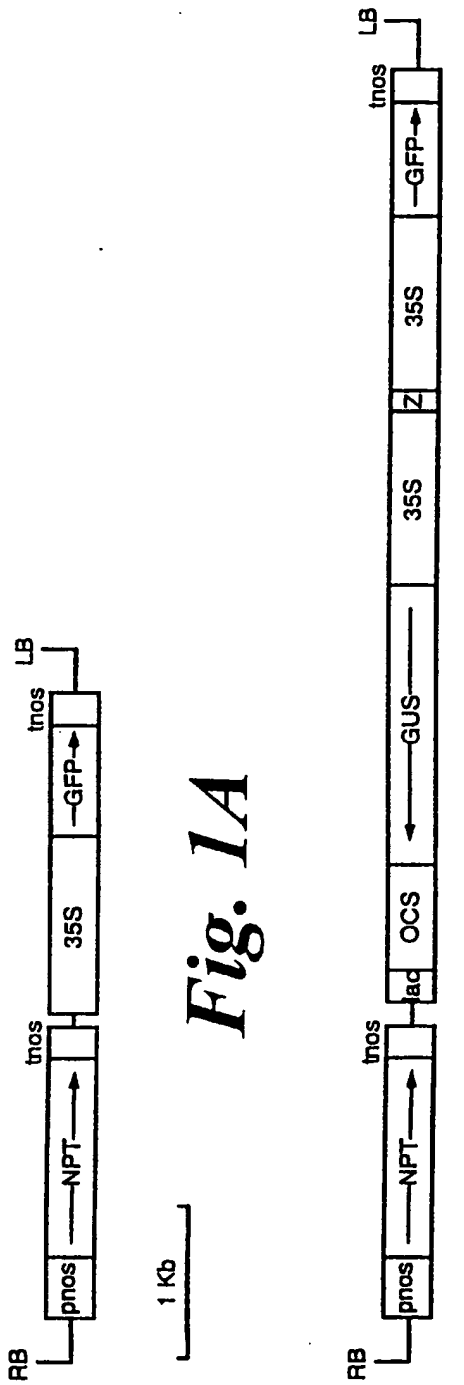
28. A method for producing a systemic gene silencing signal in a plant, said method comprising the steps of introducing a construct as claimed in any one of claims 25 to 27 into a cell of that plant.

29. A method as claimed in claim 28 wherein the signal produced by the construct is subsequently stably maintained in the absence of the construct.

30. A plant cell comprising a construct as claimed in any one of claims 25 to 27.

31. A plant comprising a plant cell as claimed in claim 30.

32. A plant comprising a target nucleotide sequence which has been silenced in accordance with the method of any one of claims 1 to 21.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RB
pnos
NPT
tnos
35S
GFP
tnos
LB
1 Kb
NPT:GUS:GFP
RB
pnos
NPT
tnos
lac
OCS
GUS
35S
Z
35S
GFP
tnos
LB
NPT:GUS
RB
pnos
NPT
tnos
35S
GUS
OCS
LB
BAR:GUS
RB
lacZ
OCS
BaR
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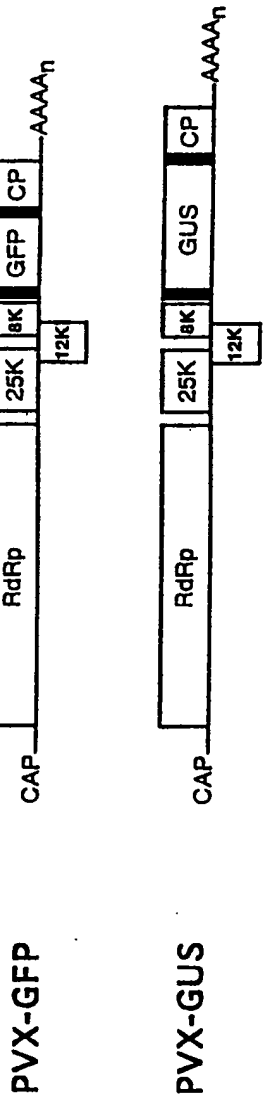
OCS
GUS
35S
LB
Vector
RB
LB
1 Kb

Fig. 1A

Fig. 1B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CAP
RdRp
25K
8K
12K
GFP
CP
AAAAn
PVX-GUS
CAP
RdRp
25K
8K
12K
GUS
CP
AAAAn
1 Kb

Fig. 1C

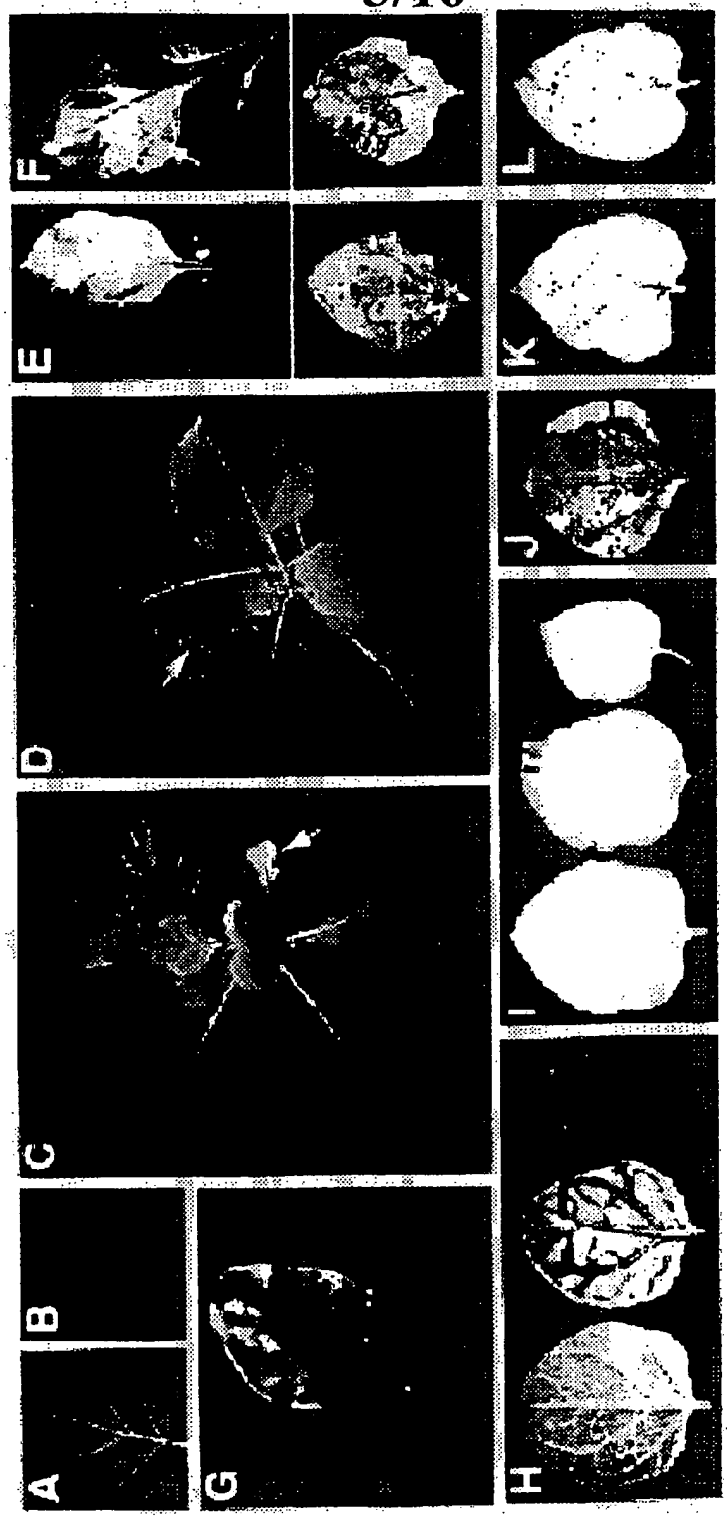


Fig. 2

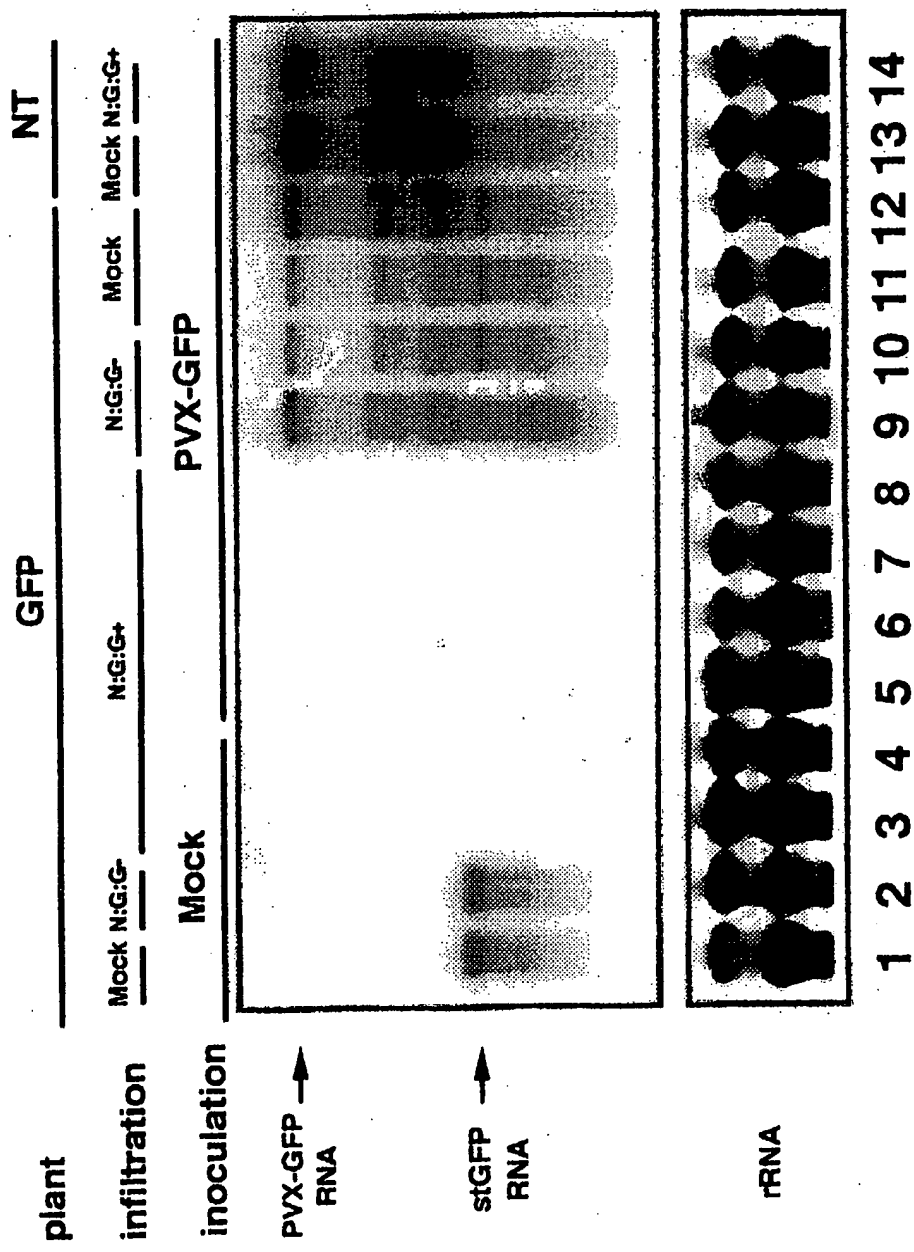
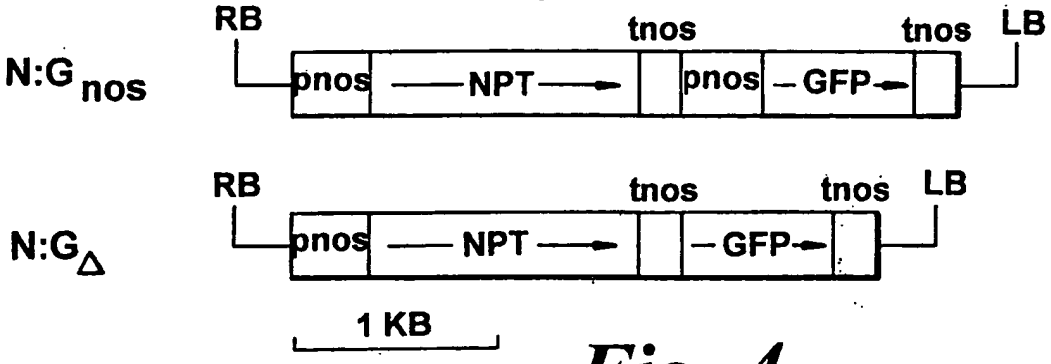

plant
GFP
NT
infiltration
Mock
N:G:G-
N:G:G+
N:G:G-
Mock
Mock
N:G:G+
inoculation
Mock
PVX-GFP
PVX-GFP RNA
stGFP RNA
rRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 3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RB
pnos
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tnos
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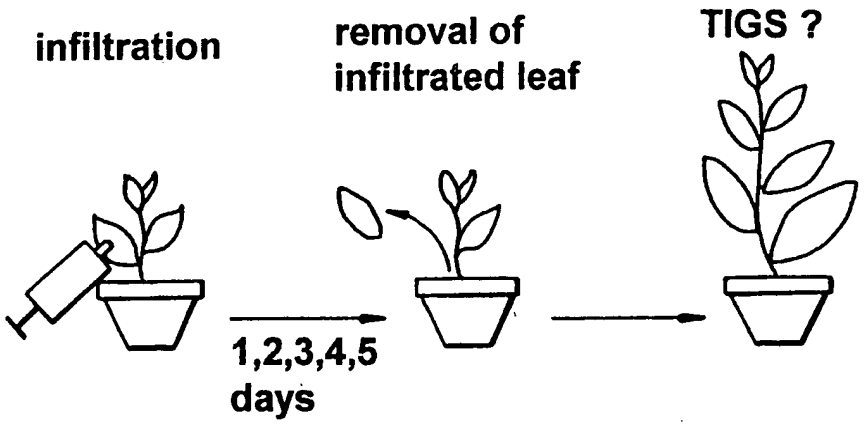
tnos
LB
1 KB

Fig. 4


infiltration
removal of infiltrated leaf
TIGS ?
1,2,3,4,5 days

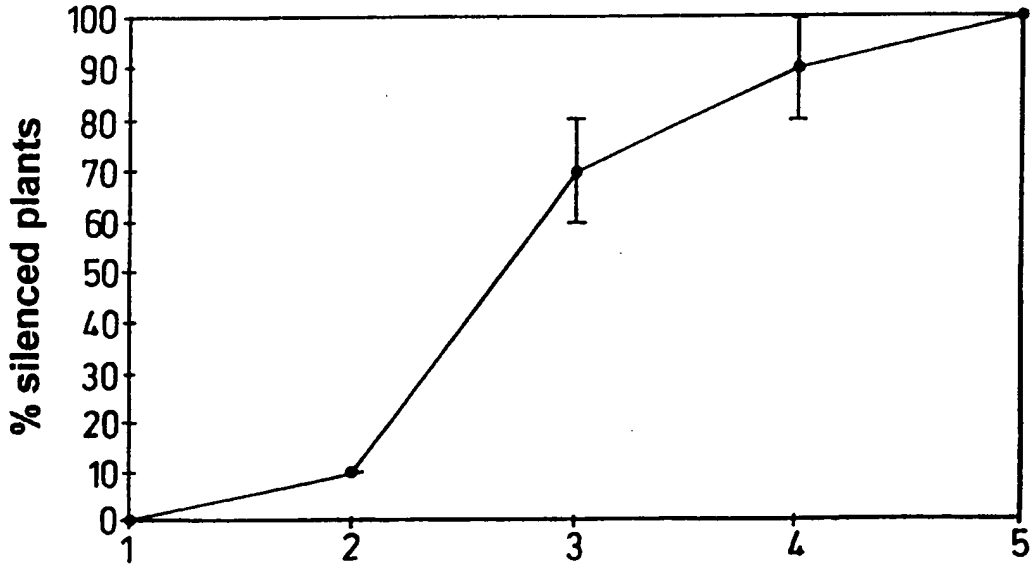

% silenced plants
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5

Fig. 5

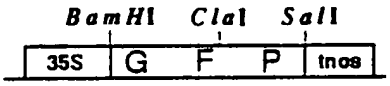
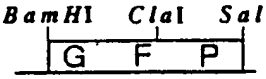
Bombarded DNA		% silenced plants	No. plants
pUC35S-GFP		75 +/- 11	70
GFP		45 +/- 12	50
..P		11 +/- 6	50
G..		32 +/- 6	50

Fig. 6A

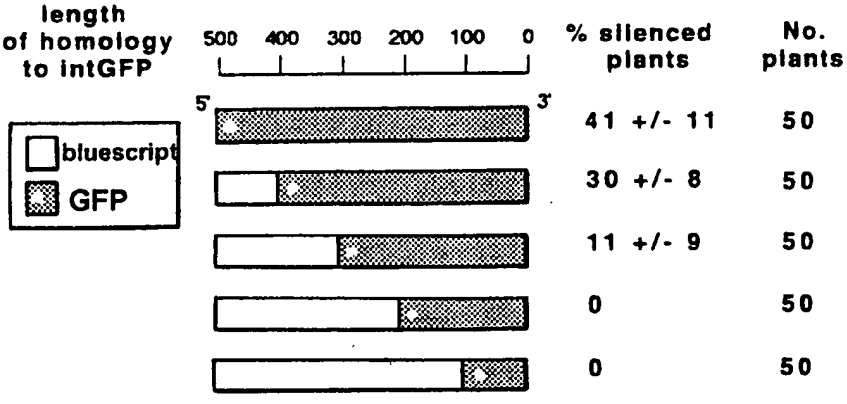


Fig. 6B

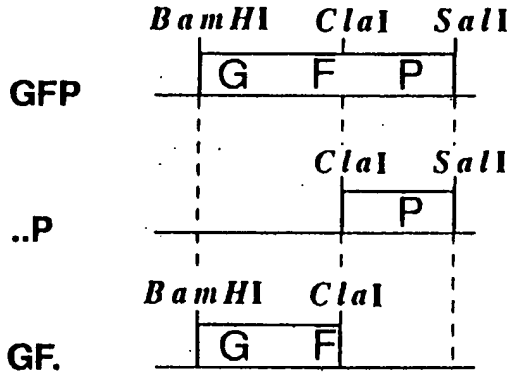
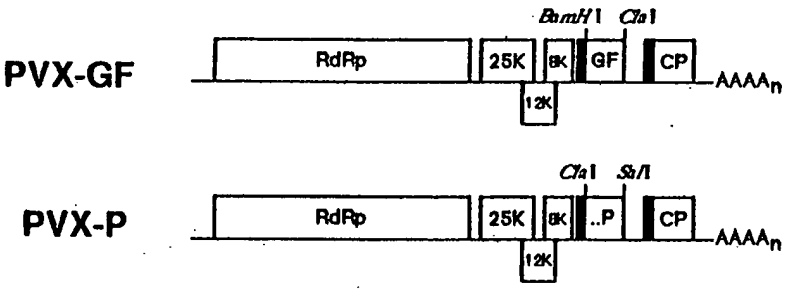
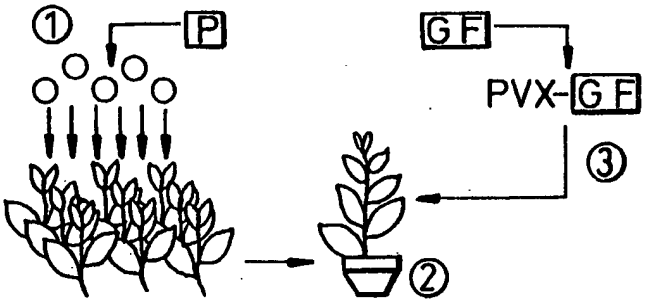

BamHI ClaI SalI
GFP
G F P
ClaI SalI
..P
P
BamHI ClaI
GF.
G F

Fig. 7A


PVX-GF
BamHI ClaI
RdRp
25K
8K
GF
CP
AAAAn
12K
PVX-P
ClaI SalI
RdRp
25K
8K
..P
CP
AAAAn
12K


①
P
GF
PVX-GF
③
②

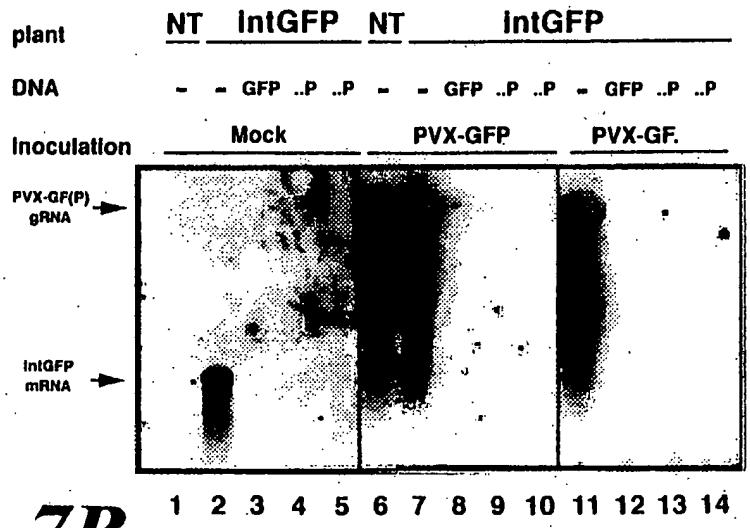

plant
NT IntGFP NT IntGFP
DNA
- - GFP ..P ..P - - GFP ..P ..P - GFP ..P ..P
Inoculation
Mock PVX-GFP PVX-GF.
PVX-GF(P) gRNA
IntGFP mRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 7B

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progenitor construct: pPVX209 (in pUC19 vector)

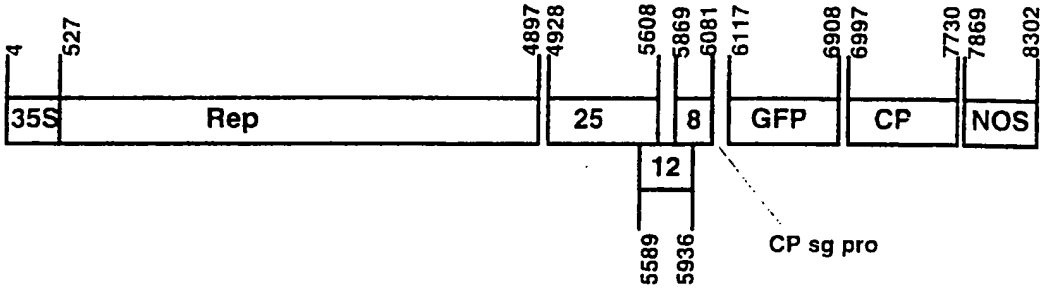
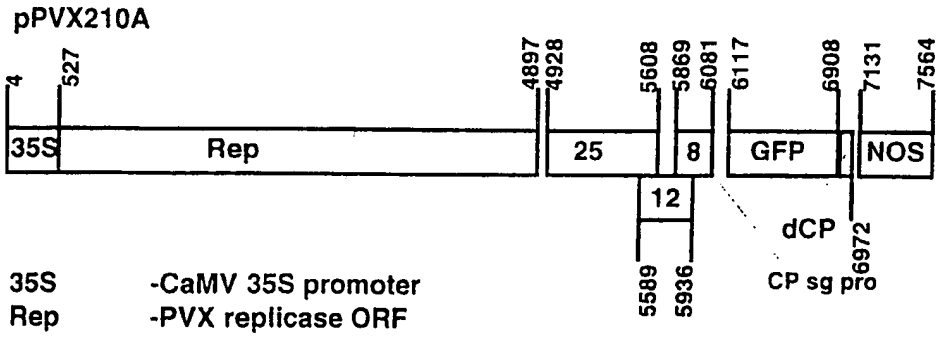


Fig. 8A



35S	-CaMV 35S promoter
Rep	-PVX replicase ORF
dRep	-5'-truncated PVX replicase ORF
25	-25-kDa ORF
d25	-5'-truncated 25-kDa ORF
12	-12-kDa ORF
8	-8-kDa ORF
GFP	-GFP5 ORF
dGFP	-5'-truncated GFP ORF
CP	-coat protein ORF
CP sg pro	-duplicated subgenomic CP RNA promoter
dCP	-5'-truncated GFP ORF
NOS	-NOS terminator

Fig. 8B

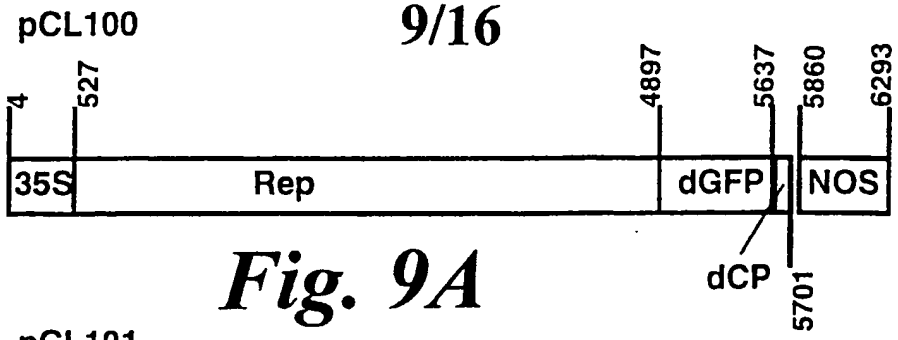


Fig. 9A

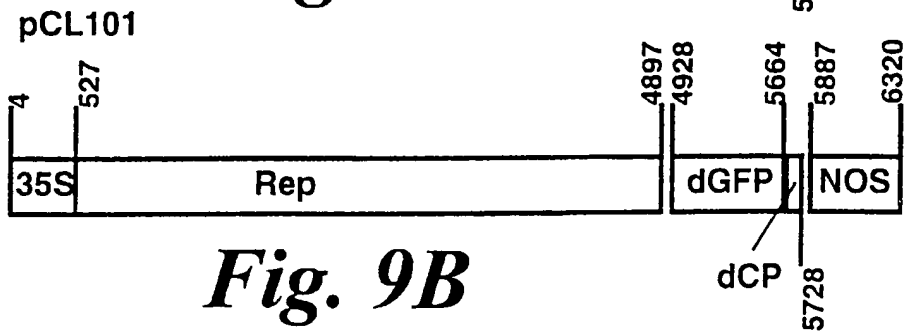


Fig. 9B

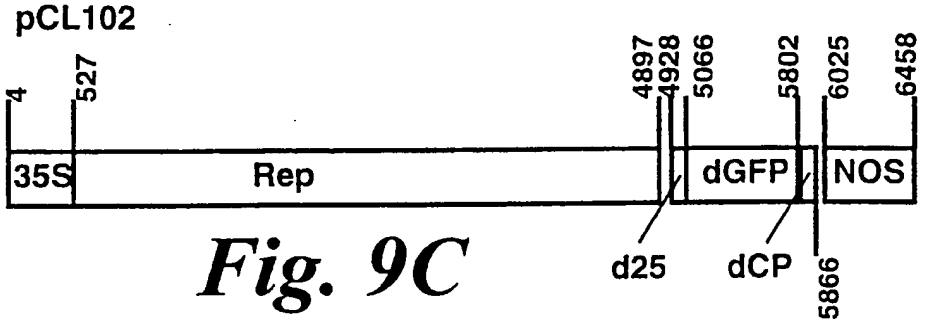


Fig. 9C

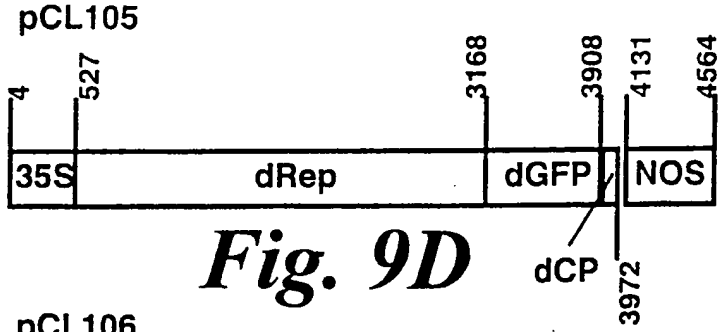


Fig. 9D

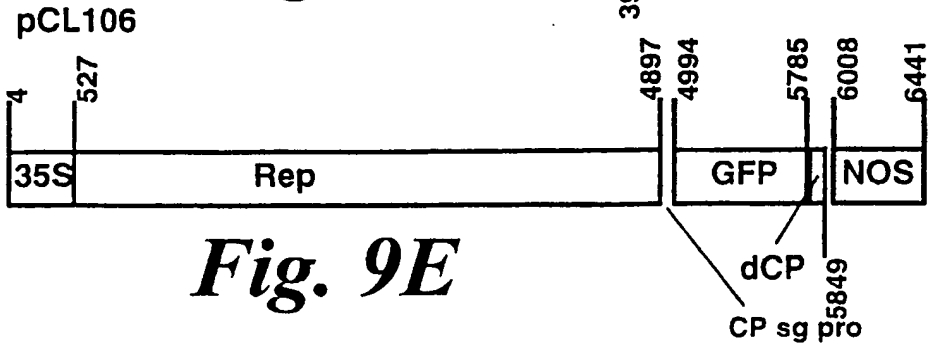


Fig. 9E

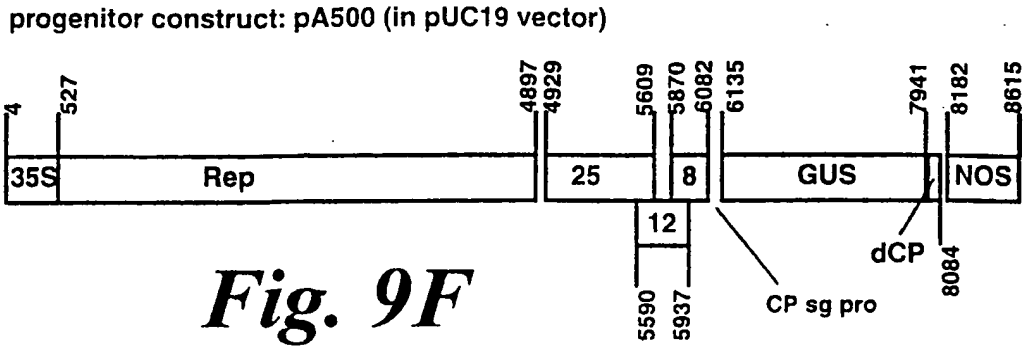


Fig. 9F

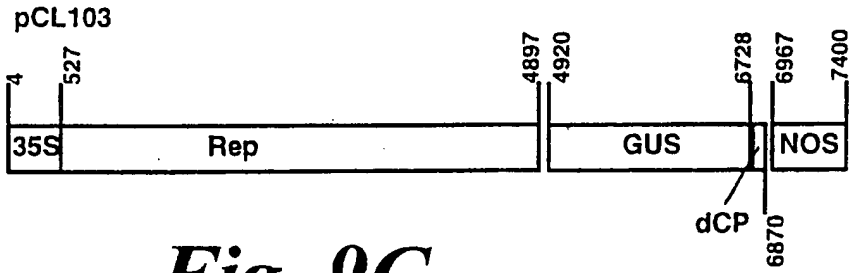


Fig. 9G

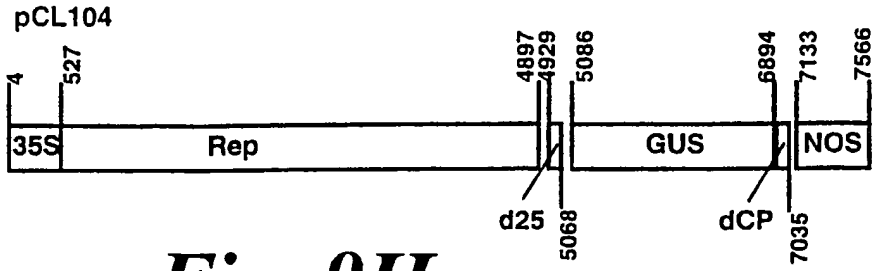


Fig. 9H

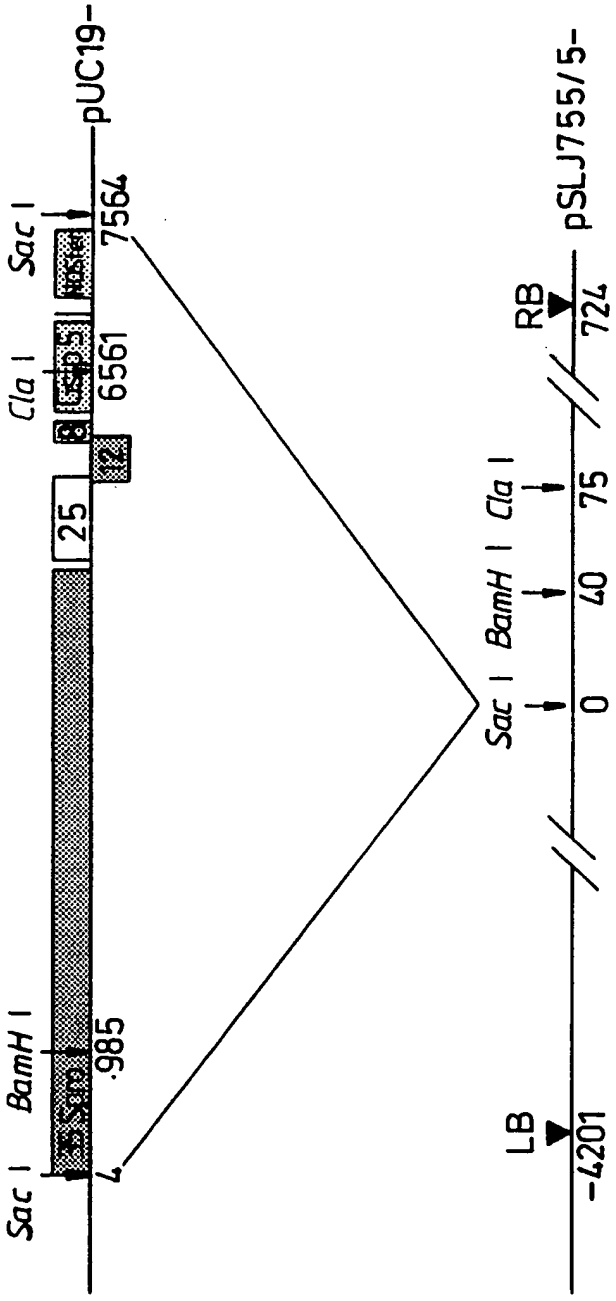

pUC19-
Sac I
7564
Nos ter
Cla I
6561
8
35S p-3
12
25
BamH I
985
35S p
Sac I
pSLJ755/5-
RB
724
Cla I
75
BamH I
40
Sac I
0
LB
-4201

Fig. 10

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~/pUC19/35Spro/replicase~GCACAGATTTTCCTAGG CACGTTATCAATTA
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CGTATCACTGGAACAACAACCGCTGAGGCTGTTGTCACTCTACCACCACCATAA~/poly(
A)/NOSter/~

Fig. 11A

13/16

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TGAAGACTAATCTTTTTCTCTTTCTCATCTTTTCACTTCTCCTATCATTATCCTCGGCCG
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GAG GT
CGTATCACTGGAACAACAACCGCTGAtGCTGTTGTCACTCTACCACCACCATAA~/poly(
A)/NOSter/~

Fig. 11B

14/16

~/pUC19/35Spro/replicase~GCACAGATTTTCCTAGG CACGTTATCAATTA
TGCGCCTGACTGGTGAAGGTCCCACTTTTGATGCAAACACTGAGTGCAACATAGCTTACAC
CCATACAAAGTTTGACATCCCAGCCGGAACTGCTCAAGTTTATGCAGGAGACGACTCCGCA
CTGGACTGTGTTCCA*t*AAGTGAAGCATAGTTTCCACAGGCTTGAGGACAAATTACTCCTAA
AGTCAAAGCCTGTAATCACGCAGCAAAAGAAGGGCAGTTGGCCTGAGTTTTGTGGTTGGCT
GATCACACCAAAAGGGGTGATGAAAGACCCAATTAAGCTCCATGTTAGCTTAAAATTGGCT
GAAGCTAAGGGTGAACTCAAGAAATGTCAAGATTCCTATGAAATTGATCTGAGTTATGCCT
ATGACCACAAGGACTCTCTGCATGACTTGTTCGATGAGAAACAGTGTCAGGCACACACACT
CACTTGCA*t*AACACTAATCAAGTCAGGGAGAGGCACTGTCTCACTTTCCCGCCTCAGAAAC
TTTCTTTAACCG

CGGCCG
AATT~GFP5sequence~ACATGACGAACTCTAAATGTCGAG GTCGTATCACTG
GAACAACAACCGCTGATGCTGTTGTCACTCTACCACCACCATAA~/poly(A)/NOSter/
~

Fig. 11C

15/16

~/pUC19/35Spro/replicase~GCACAGATTTTCCTAGG CACGTTATCAATTA
TGCGCCTGACTGGTGAAGGTCCCACTTTTGATGCAAACACTGAGTGCAACATAGCTTACAC
CCATACAAAGTTTGACATCCCAGCCGGAACTGCTCAAGTTTATGCAGGAGACGACTCCGCA
CTGGACTGTGTTCCA*t*AAGTGAAGCATAGTTTCCACAGGCTTGAGGACAAATTACTCCTAA
AGTCAAAGCCTGTAATCACGCAGCAAAAGAAGGGCAGTTGGCCTGAGTTTTGTGGTTGGCT
GATCACACCAAAAGGGGTGATGAAAGACCCAATTAAGCTCCATGTTAGCTTAAAATTGGCT
GAAGCTAAGGGTGAACTCAAGAAATGTCAAGATTCCTATGAAATTGATCTGAGTTATGCCT
ATGACCACAAGGACTCTCTGCATGACTTGTTCGATGAGAAACAGTGTCAGGCACACACACT
CACTTGCAGAACACTAATCAAGTCAGGGAGAGGCACTGTCTCACTTTCCCGCCTCAGAAAC
TTTCTTTAACCGTTAAGTTACCTTAGAGATTTGAATAAGATGGATATTCTCATCAGTAGTT
TGAAAAGTTTAGGTTATTCTAGGACTTCCAAATCTTTAGATTCAGGACCTTTGGTAGTACA
TGCAGTAGCCGGAGCCGGTAAGTCCACAGCCCTAAGGAAGTTGATCCTCAGACAC_____
_____
_____
CGGCCG
AATT~GFP5sequence~ACATGACGAACTCTAAATGTCGAG GTCGTATCACTG
GAACAACAACCGCTGATGCTGTTGTCACTCTACCACCACCATAA~/poly(A)/NOSter/
~

Fig. 11D

~/pUC19/35Spro/replicase~GCACAGATTTTCCTAGG CACGTTATCAATTA
TGCGCCTGACTGGTGAAGGTCCCACTTTTGATGCAAACACTGAGTGCAACATAGCTTACAC
CCATACAAAGTTTGACATCCCAGCCGGAACTGCTCAAGTTTATGCAGGAGACGACTCCGCA
CTGGACTGTGTTCCA*t*AAGTGAAGCATAGTTTCCACAGGCTTGAGGACAAATTACTCCTAA
AGTCAAAGCCTGTAATCACGCAGCAAAAGAAGGGCAGTTGGCCTGAGTTTTGTGGTTGGCT
GATCACACCAAAAGGGGTGATGAAAGACCCAATTAAGCTCCATGTTAGCTTAAAATTGGCT
GAAGCTAAGGGTGAACTCAAGAAATGTCAAGATTCCTATGAAATTGATCTGAGTTATGCCT
ATGACCACAAGGACTCTCTGCATGACTTGTTCGATGAGAAACAGTGTCAGGCACACACACT
CACTTGCAGAACACTAATCAAGTCAGGGAGAGGCACTGTCTCACTTTCCCGCCTCAGAAAC
TTTCTTTAACCGTTAAGTTACCTTAGAGATTTGAATAAG_____
_____
_____
_____
_____
CGGCCG
AATT~GFP5sequence~ACATGACGAACTCTAAATGTCGAG GTCGTATCACTG
GAACAACAACCGCTGATGCTGTTGTCACTCTACCACCACCATAA~/poly(A)/NOSter/
~

Fig. 11E

16/16

~/pUC19/35Spro/replicase~GCACAGATTTTCCTAGG CACGTTATCAATTA
TGCGCCTGACTGGTGAAGGTCCCACTTTTGATGCAAACACTGAGTGCAACATAGCTTACAC
CCATACAAAGTTTGACATCCCAGCCGGAACTGCTCAAGTTTATGCAGGAGACGACTCCGCA
CTGGACTGTGTTCCAGAAGTGAAGCATAGTTTCCACAGGCTTGAGGACAAATTACTCCTAA
AGTCAAAGCCTGTAATCACGCAGCAAAAGAAGGGCAGTTGGCCTGAGTTTTGTGGTTGGCT
GATCACACCAAAAGGGGTGATGAAAGACCCAATTAAGCTCCATGTTAGCTTAAAATTGGCT
GAAGCTAAGGGTGAACTCAAGAAATGTCAAGATTCCTATGAAATTGATCTGAGTTATGCCT
ATGACCACAAGGACTCTCTGCATGACTTGTTCGATGAGAAACAGTGTCAGGCACACACACT
CACTTGCAGAACACTAATCAAGTCAGGGAGAGGCACTGTCTCACTTTCCCGCCTCAGAAAC
TTTCTTTAACCGctagcGGGCCATTGCCGATCTCAAGCCACTCTCCGTTGAACGGTTAAGT
TTCCATTGATACTCGAAAGAGGTCAGCACCAGCTAGCATCGGACATGAAGACTAATCTTTT
TCTCTTTCTCATCTTTTCACTTCTCCTATCATTATCCT_____
_____
_____
_____CGGCCG
AATT~GFP5sequence~ACATGACGAACTCTAAATGTCGAG GTCGTATCACTG
GAACAACAACCGCTGATGCTGTTGTCACTCTACCACCACCATAA~/poly(A)/NOSter/
~

Fig. 11F

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(71) Applicants *(for all designated States except US)*: THE CARNEGIE INSTITUTE OF WASHINGTON [US/US]; 1530 P Street, N.W., Washington, DC 20005 (US). THE UNIVERSITY OF MASSACHUSETTS [US/US]; One Beacon Street, Boston, MA 02108 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: FIRE, Andrew [US/US]; 2320 Bright Leaf Way, Baltimore, MD 21210 (US). XU, Siqun [CN/US]; 1755 Warminton Court, Ballwin, MO 63021 (US). MONTGOMERY, Mary, K. [US/US]; 233 Macalester Street, St. Paul, MN 55105 (US). KOSTAS, Stephen, A. [US/US]; 126 East Melrose Avenue, Baltimore, MD 21212 (US). TIMMONS, Lisa [US/US]; 2408 Brambleton Road, Baltimore, MD 21209 (US). TABARA, Hiroaki [JP/US]; Apartment #1, 145 Orient Street, Worcester, MA 01604 (US). DRIVER, Samuel, E. [US/US]; Apartment #4, 1714 Commonwealth Avenue, Brighton, MA 02135 (US). MELLO, Craig, C. [US/US]; 19 Ryan Road, Shrewsbury, MA 01545 (US).

(74) Agents: KOKULIS, Paul, N. et al.; Pillsbury Madison & Sutro LLP, 1100 New York Avenue, N.W., Washington, DC 20005 (US).

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(54) Title: GENETIC INHIBITION BY DOUBLE-STRANDED RNA

(57) Abstract

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced *ex vivo* or *in vivo*. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

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GENETIC INHIBITION BY DOUBLE-STRANDED RNA

GOVERNMENT RIGHTS

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BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a *directed* change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based, for example: cases in which is important to

produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stochiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule *in vitro* under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use *in vivo* has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells *in vivo*. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and *Drosophila*. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been

hypothesized to occur; an as-yet-unidentified mechanism would then lead to *de novo* methylation and subsequent suppression of gene expression. Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering RNA, as well as the nature of the interference process, have not been determined. Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous gene. Initial results with this technique have been encouraging, however, the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

Distinction between the Present Invention and Antisense Approaches

The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by double-stranded RNA must occur by a mechanism distinct from antisense interference.

Distinction between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated

inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has been used to inhibit expression of 18 different genes, providing phenocopies of null mutations in these genes of known function. The extreme environmental and sequence constraints on triple-helix formation make it unlikely that dsRNA-mediated inhibition *in C. elegans* is mediated by a triple-strand structure.

Distinction between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and *Drosophila* indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be

defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or in *vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region may be used to transcribe the RNA strand (or strands).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The advantages of the present invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA which can be used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of antisense interference. This invention is not limited to *in vitro* use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, this invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. These concerns have been a serious obstacle to designing general strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease

models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; *unc-22* [9], *unc-54* [12], *fem-1* [14], and *hlh-1* [15]).

Figures 2 A-I show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins, nuclear GFP-LacZ and mitochondrial GFP. The micrographs show progeny of injected animals visualized by a fluorescence microscope. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (ds-*unc22A*). Panels D-F show progeny of animals injected with ds-*gfpG*. Panels G-I demonstrate specificity. Animals are injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20 μm.

Figures 3 A-D show effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA. Micrographs show *in situ* hybridization to embryos (dark stain). Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous *mex-3* RNA[20]). Panel C: Embryo from a parent injected with purified *mex-3*B antisense RNA. These embryos and the parent animals retain the *mex-3* mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to *mex-3*B; no *mex-3* RNA was detected. Scale: each embryo is approximately 50 μm in length.

Figure 4 shows inhibitory activity of *unc-22A* as a function of structure and concentration. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included. Progeny cohort groups are labeled **1** for 0-6 hours, **2** for 6-15

hours, 3 for 15-27 hours, 4 for 27-41 hours, and 5 for 41-56 hours The bottom-left diagram shows genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids[8,3].

Figures 5 A-C show examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. Panel A: General strategy for production of dsRNA by cloning a segment of interest between flanking copies of the bacteriophage T7 promoter and transcribing both strands of the segment by transfecting a bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter. Panel B: A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), fed on a native bacterial host. Panel C: PD4251 animals reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing sequence-specific inhibition of gene expression by introducing double-stranded RNA (dsRNA). A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell. Inhibition is sequence-specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen which is capable of infecting an organism from which the cell is derived. Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The

consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

The RNA may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored

to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by *in vitro* enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, *Sequence Analysis Primer*, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C hybridization for 12-16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25. 50, 100, 200, 300 or 400 bases.

As disclosed herein, 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of

being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative generae of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhchonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphalenchus, Criconemella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be synthesized either *in vivo* or *in vitro*. Endogenous RNA polymerase of the cell may mediate transcription *in vivo*, or cloned RNA polymerase can be used for transcription *in vivo* or in *vitro*. For transcription from a transgene *in vivo* or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art[32, 33, 34] (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by *in vitro* enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively, the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, intro-

duced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression. Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced. A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

The present invention may be used to introduce RNA into a cell for the treatment or prevention of disease. For example, dsRNA may be introduced into a cancerous cell or tumor and thereby inhibit gene expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation or maintenance of the disease/pathology.

Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. The inhibitory RNA could be introduced in cells *in vitro* or *ex vivo* and then subsequently placed into an animal to affect therapy, or directly treated by *in vivo* administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of prophylaxis (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, cranio-pharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adeno-

carcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, digestive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized or transformed. The invention could be used in combination with other treatment modalities, such as chemotherapy, cryotherapy, hyperthermia, radiation therapy, and the like.

As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases,

amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

The present invention could comprise a method for producing plants with reduced susceptibility to climatic injury, susceptibility to insect damage, susceptibility to infection by a pathogen, or altered fruit ripening characteristics. The targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). If an expression construct is used to transcribe the RNA in a plant, transcription by a wound- or stress-inducible; tissue-specific (e.g., fruit, seed, anther, flower, leaf, root); or otherwise regulatable (e.g., infection, light, temperature, chemical) promoter may be used. By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in *Index of Plant Diseases in the United States* (U.S. Dept. of Agriculture Handbook No. 165, 1960); *Distribution of Plant-Parasitic Nematode Species in North America* (Society of Nematologists, 1985); and *Fungi on Plants and Plant Products in the United States* (American Phytopathological Society, 1989). Insects with reduced ability to damage crops or improved ability to prevent other destructive insects from damaging crops may be produced. Furthermore, some nematodes are vectors of plant pathogens, and may be attacked by other beneficial nematodes which have no effect on plants. Inhibition of target gene activity could be used to delay or

prevent entry into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle. Interactions between pathogens may also be modified by the invention to limit crop damage. For example, the ability of beneficial nematodes to attack their harmful prey may be enhanced by inhibition of behavior-controlling nematode genes according to the invention.

Although pathogens cause disease, some of the microbes interact with their plant host in a beneficial manner. For example, some bacteria are involved in symbiotic relationships that fix nitrogen and some fungi produce phytohormones. Such beneficial interactions may be promoted by using the present invention to inhibit target gene activity in the plant and/or the microbe.

Another utility of the present invention could be a method of identifying gene function in an organism comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (e.g., nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which RNA can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96-well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by *in vivo* or *in vitro* transcription from an expression construct used to produce the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example: arabidopsis, bacteria, drosophila, fungi, nematodes, viruses, zebrafish, and tissue culture cells derived from mammals.

A nematode or other organism that produces a colorimetric, fluorogenic, or luminescent signal in response to a regulated promoter (e.g., transfected with a reporter gene construct) can be assayed in an HTS format to identify DNA-binding proteins that regulate the promoter. In the assay's simplest form, inhibition of a negative regulator results in an increase of the signal and inhibition of a positive regulator results in a decrease of the signal.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the

characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Pesticides may include the RNA molecule itself, an expression construct capable of expressing the RNA, or organisms transfected with the expression construct. The

pesticide of the present invention may serve as an arachnicide, insecticide, nematicide, viricide, bactericide, and/or fungicide. For example, plant parts that are accessible above ground (e.g., flowers, fruits, buds, leaves, seeds, shoots, bark, stems) may be sprayed with pesticide, the soil may be soaked with pesticide to access plant parts growing beneath ground level, or the pest may be contacted with pesticide directly. If pests interact with each other, the RNA may be transmitted between them. Alternatively, if inhibition of the target gene results in a beneficial effect on plant growth or development, the aforementioned RNA, expression construct, or transfected organism may be considered a nutritional agent. In either case, genetic engineering of the plant is not required to achieve the objectives of the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Used as either an pesticide or nutrient, a formulation of the present invention may be delivered to the end user in dry or liquid form: for example, as a dust, granulate, emulsion, paste, solution, concentrate, suspension, or encapsulation. Instructions for safe and effective use may also be provided with the formulation. The formulation might be used directly, but concentrates would require dilution by mixing with an extender provided by the formulator or the end user. Similarly, an emulsion, paste, or suspension may require the end user to perform certain preparation steps before application. The formulation may include a combination of chemical additives known in the art such as solid carriers, minerals, solvents, dispersants, surfactants, emulsifiers, tackifiers, binders, and other adjuvants. Preservatives and stabilizers may also be added to the formulation to facilitate storage. The crop area or plant may also be treated simultaneously or separately with other pesticides or fertilizers. Methods of application include dusting, scattering or pouring, soaking, spraying, atomizing, and coating. The precise physical form and chemical composition of the formulation, and its method of application, would be chosen to promote the objectives of the invention and in accordance with prevailing circumstances. Expression constructs and transfected hosts capable of replication may also promote the persistence and/or spread of the formulation.

Description of the dsRNA Inhibition Phenomenon in *C. elegans*

The operation of the present invention was shown in the model genetic organism *Caenorhabditis elegans*.

Introduction of RNA into cells had been seen in certain biological systems to interfere with function of an endogenous gene[1,2]. Many such effects were believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism had been suggested. One instance of an RNA-mediated mechanism was RNA interference (RNAi) phenomenon in the nematode *C. elegans*. RNAi had been used in a variety of studies to manipulate gene expression[3,4].

Despite the usefulness of RNAi in *C. elegans*, many features had been difficult to explain. Also, the lack of a clear understanding of the critical requirements for interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the literature was that sense and antisense RNA preparations are each sufficient to cause interference[3,4]. The only precedent for such a situation was in plants where the process of co-suppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give effective and uniform genetic inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of crude sense and antisense preparations to produce interference[3,4] had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

The inventors carefully purified sense or antisense RNA for *unc-22* and tested each for gene-specific inhibition. While the crude sense and antisense preparations had strong interfering activity, it was found that the purified sense and antisense RNAs had only marginal inhibitory activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific *in vitro*

promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters *in vivo*, is produced predominantly from a single strand. The inventors had carried out purification of these crude preparations to investigate whether a small fraction of the RNA had an unusual structure which might be responsible for the observed genetic inhibition. To rigorously test whether double-stranded character might contribute to genetic inhibition, the inventors carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

Analysis of RNA-Mediated Inhibition of *C. elegans* Genes

The *unc-22* gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between *unc-22* gene activity and the movement phenotypes of animals[3,8]: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. *unc-22* encodes an abundant but non-essential myofilament protein[7-9]. *unc-22* mRNA is present at several thousand copies per striated muscle cell[3].

Purified antisense and sense RNAs covering a 742 nt segment of *unc-22* had only marginal inhibitory activity, requiring a very high dose of injected RNA for any observable effect (Figure 4). By contrast, a sense+antisense mixture produced a highly effective inhibition of endogenous gene activity (Figure 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The lowest dose of the sense+antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. *unc-22* expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense+antisense mixture could reflect formation of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predomi-

nantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands, were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism[10]. Conceivably, the inventive sense+antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. This was not found to be the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of *unc-22* single strands to mediate inhibition. Also investigated was whether double-stranded structure could potentiate inhibitory activity when placed in *cis* to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded *unc-22* segment did not stimulate inhibition. Thus potentiation of gene-specific inhibition was observed only when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by *unc-22* dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic *unc-22* loss of function mutants. Target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (Figure 1 and Table 1). *unc-54* encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction[7,11,12], *fem-1* encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production[13,14], and *hlh-1* encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility[15,16]. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment *unc54C*, which led to an embryonic and

larval arrest phenotype not seen with *unc-54* null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to inhibit the activity of other highly related myosin heavy chain genes[17]. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The *unc-54* and *hlh-1* muscle phenotypes, in particular, are known to result from a large number of defective muscle cells[11,16]. To examine inhibitory effects of dsRNA on a cellular level, a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle was used. Injection of dsRNA directed to *gfp* produced dramatic decreases in the fraction of fluorescent cells (Figure 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with *gfp* inhibition was not random. At low doses of dsRNA, the inventors saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13-14 versus 8-9 for embryonic muscles[18,19]). At high concentrations of *gfp* dsRNA, inhibition was noted in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or post-embryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans*:

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, dsRNA injection produced a dramatic decrease in the level of the endogenous mRNA transcript (Figure 3). Here, a *mex-3* transcript that is abundant in the gonad and early embryos[20] was targeted, where straightforward *in situ* hybridization can be performed[5]. No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3* (Figure 3D), but injection of purified *mex-3* antisense RNA resulted in animals that retained substantial endogenous mRNA levels (Figure 3C).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for *unc-22*, *gfp*, or *lacZ* into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient "nicking" of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

Table 3 shows that *C. elegans* can respond in a gene-specific manner to dsRNA encountered in the environment. Bacteria are a natural food source for *C. elegans*. The bacteria are ingested, ground in the animal's pharynx, and the bacterial contents taken up in the gut. The results show that *E. coli* bacteria expressing dsRNAs can confer specific inhibitory effects on *C. elegans* nematode larvae that feed on them.

Three *C. elegans* genes were analyzed. For each gene, corresponding dsRNA was expressed in *E. coli* by inserting a segment of the coding region into a plasmid construct designed for bidirectional transcription by bacteriophage T7 RNA polymerase. The dsRNA segments used for these experiments were the same as those used in previous microinjection experiments (see Figure 1). The effects resulting from feeding these bacteria to *C. elegans* were compared to the effects achieved by microinjecting animals

with dsRNA.

The *C. elegans* gene *unc-22* encodes an abundant muscle filament protein. *unc-22* null mutations produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction. When wild-type animals were fed bacteria expressing a dsRNA segment from *unc-22*, a high fraction (85%) exhibited a weak but still distinct twitching phenotype characteristic of partial loss of function for the *unc-22* gene. The *C. elegans fem-1* gene encodes a late component of the sex determination pathway. Null mutations prevent the production of sperm and lead euploid (XX) animals to develop as females, while wild type XX animals develop as hermaphrodites. When wild-type animals were fed bacteria expressing dsRNA corresponding to *fem-1*, a fraction (43%) exhibit a sperm-less (female) phenotype and were sterile. Finally, the ability to inhibit gene expression of a transgene target was assessed. When animals carrying a *gfp* transgene were fed bacteria expressing dsRNA corresponding to the *gfp* reporter, an obvious decrease in the overall level of GFP fluorescence was observed, again in approximately 12% of the population (see Figure 5, panels B and C).

The effects of these ingested RNAs were specific. Bacteria carrying different dsRNAs from *fem-1* and *gfp* produced no twitching, dsRNAs from *unc-22* and *fem-1* did not reduce *gfp* expression, and dsRNAs from *gfp* and *unc-22* did not produce females. These inhibitory effects were apparently mediated by dsRNA: bacteria expressing only the sense or antisense strand for either *gfp* or *unc-22* caused no evident phenotypic effects on their *C. elegans* predators.

Table 4 shows the effects of bathing *C. elegans* in a solution containing dsRNA. Larvae were bathed for 24 hours in solutions of the indicated dsRNAs (1 mg/ml), then allowed to recover in normal media and allowed to grow under standard conditions for two days. The *unc-22* dsRNA was segment ds-*unc22A* from Figure 1. *pos*-1 and *sqt*-3 dsRNAs were from the full length cDNA clones. *pos-1* encodes an essential maternally provided component required early in embyogenesis. Mutations removing *pos-1* activity have an early embryonic arrest characteristic of *skn*-like mutations[29, 30]. Cloning and activity patterns for *sqt-3* have been described[31]. *C. elegans sqt-3* mutants have mutations in the *col-1* collagen gene[31]. Phenotypes of affected animals are noted. Incidences of

clear phenotypic effects in these experiments were 5-10% for *unc-22*, 50% for *pos-1*, and 5% for *sqt-3*. These are frequencies of unambiguous phenocopies; other treated animals may have had marginal defects corresponding to the target gene that were not observable. Each treatment was fully gene-specific in that *unc-22* dsRNA produced only Unc-22 phenotypes, *pos-1* dsRNA produced only Pos-1 phenotypes, and *sqt-3* dsRNA produced only Sqt-3 phenotypes.

Some of the results described herein were published after the filing of our provisional application. Those publications and a review can be cited as Fire, A., et al. Nature, 391, 806-811, 1998; Timmons, L. & Fire, A. Nature, 395, 854, 1998; and Montgomery, M.K. & Fire, A. Trends in Genetics, 14, 255-258, 1998.

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions[21] for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, one case was observed in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family.

Methods of RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase[6], followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original "sense" and "antisense" preparations. Nonetheless, RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant inhibition. This inhibitory activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Following a short (5 minute) treatment at 68°C to remove secondary structure, sense+antisense annealing was carried out in injection buffer[27] at 37°C for 10-30 minutes.

Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA *in vitro*. Non-annealed sense+antisense RNAs for *unc22B* and *gfpG* were tested for inhibitory effect and found to be much more active than the individual single strands, but 2-4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for *unc22A*, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of 0.5×10^6 to 1.0×10^6 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA (i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

Methods for Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with *gfp* [27] and *lacZ* activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccIs4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP), pSAK2 (*myo-3* promoter driving a nuclear targeted GFP-LacZ fusion), and a *dpy-20* subclone[26] as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between

cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the microinjection needle into the gonadal syncitium of adults and expelling 20-100 pl of solution (see Reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype. After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

Additional Description of the Results

Figure 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; sequence references are as follows: *unc-22* [9], *unc-54* [12], *fem-1* [14], and *hlh-1* [15]). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for inhibitory effects is designated with the name of the gene followed by a single letter (e.g., *unc22C*). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

The effects of inhibitory RNA were analyzed in individual cells (Figure 2, panels A-H). These experiments were carried out in a reporter strain (called PD4251) expressing

two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene. ds-*unc22A* RNA was injected as a negative control. GFP expression in progeny of these injected animals was not affected. The GFP patterns of these progeny appeared identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP): young larva (Figure 2A), adult (Figure 2B), and adult body wall at high magnification (Figure 2C).

In contrast, the progeny of animals injected with ds-*gfpG* RNA are affected (Figures 2D-F). Observable GFP fluorescence is completely absent in over 95% of the cells. Few active cells were seen in larvae (Figure 2D shows a larva with one active cell; uninjected controls show GFP activity in all 81 body wall muscle cells). Inhibition was not effective in all tissues: the entire vulval musculature expressed active GFP in an adult animal (Figure 2E). Rare GFP positive body wall muscle cells were also seen adult animals (two active cells are shown in Figure 2F). Inhibition was target specific (Figures 2G-I). Animals were injected with ds-*lacZL* RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appeared unaffected while the nuclear-targeted GFP-LacZ was absent from almost all cells (larva in Figure 2G). A typical adult lacked nuclear GFP-LacZ in almost all body-wall muscles but retained activity in vulval muscles (Figure 2H). Scale bars in Figure 2 are 20 μm.

The effects of double-stranded RNA corresponding to *mex-3* on levels of the endogenous mRNA was shown by *in situ* hybridization to embryos (Figure 3, panels A-D). The 1262 nt *mex-3* cDNA clone[20] was divided into two segments, *mex-3*A and *mex-3*B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. *mex-3*B antisense or dsRNA was injected into the gonads of adult animals, which were maintained under standard culture conditions for 24 hours before fixation and *in situ* hybridization (see Reference 5). The *mex-3*B dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to *mex-3*A were used to

assay distribution of the endogenous *mex-3* mRNA (dark stain). Four-cell stage embryos were assayed; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults. The negative control (the absence of hybridization probe) showed a lack of staining (Figure 3A). Embryos from uninjected parents showed a normal pattern of endogenous *mex-3* RNA (Figure 3B). The observed pattern of *mex-3* RNA was as previously described in Reference 20. Injection of purified *mex-3*B antisense RNA produced at most a modest effect: the resulting embryos retained *mex-3* mRNA, although levels may have been somewhat less than wild type (Figure 3C). In contrast, no *mex-3* RNA was detected in embryos from parents injected with dsRNA corresponding to *mex-3*B (Figure 3D). The scale of Figure 3 is such that each embryo is approximately 50 μm in length.

Gene-specific inhibitory activity by *unc-22A* RNA was measured as a function of RNA structure and concentration (Figure 4). Purified antisense and sense RNA from *unc22A* were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (*gfpG*). Injected animals were transferred to fresh culture plates 6 hours (columns labeled **1**), 15 hours (columns labeled **2**), 27 hours (columns labeled **3**), 41 hours (columns labeled **4**), and 56 hours (columns labeled **5**) after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. The bottom-left diagram shows the genetically derived relationship between *unc-22* gene dosage and behavior based on analyses of *unc-22* heterozygotes and polyploids[8,3].

Figures 5 A-C show a process and examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. A general strategy for production of dsRNA is to clone segments of interest between flanking copies of the bacteriophage T7 promoter into a bacterial plasmid construct (Figure 5A). A bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter was used as a host. A nuclease-resistant dsRNA was detected in lysates of transfected

bacteria. Comparable inhibition results were obtained with the two bacterial expression systems. A GFP-expressing *C. elegans* strain, PD4251 (see Figure 2), was fed on a native bacterial host. These animals show a uniformly high level of GFP fluorescence in body muscles (Figure 5B). PD4251 animals were also reared on a diet of bacteria expressing dsRNA corresponding to the coding region for *gfp*. Under the conditions of this experiment, 12% of these animals showed dramatic decreases in GFP (Figure 5C). As an alternative strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either *unc-22* or *gfp*. This was comparably effective.

All references (e.g., books, articles, applications, and patents) cited in this specification are indicative of the level of skill in the art and their disclosures are incorporated herein in their entirety.

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Table 1. Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

Gene and Segment		Size	Injected RNA	F1 Phenotype
unc-22			***unc-22* null mutants: strong twitchers**[7,8]	
unc22A[a]	exon 21-22	742	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22B	exon 27	1033	sense	wild type
			antisense	wild type
			sense+antisense	strong twitchers (100%)
unc22C	exon 21-22[b]	785	sense+antisense	strong twitchers (100%)
fem-1			***fem-1* null mutants: female (no sperm)**[13]	
fem1A	exon 10[c]	531	sense	hermaphrodite (98%)
			antisense	hermaphrodite (>98%)
			sense+antisense	female (72%)
fem1B	intron 8	556	sense+antisense	hermaphrodite (>98%)
unc-54			***unc-54* null mutants: paralyzed**[7,11]	
unc54A	exon 6	576	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54B	exon 6	651	sense	wild type (100%)
			antisense	wild type (100%)
			sense+antisense	paralyzed (100%)[d]
unc54C	exon 1-5	1015	sense+antisense	arrested embryos and larvae (100%)
unc54D	promoter	567	sense+antisense	wild type (100%)
unc54E	intron 1	369	sense+antisense	wild type (100%)
unc54F	intron 3	386	sense+antisense	wild type (100%)

Table 1 (continued).

Gene and Segment		Size	Injected RNA	F1 Phenotype
hlh-1			***hlh-1* null mutants: lumpy-dumpy larvae**[16]	
hlh1A	exons 1-6	1033	sense	wild type (<2% lpy-dpy)
			antisense	wild type (<2% lpy-dpy)
			sense+antisense	lpy-dpy larvae (>90%)[e]
hlh1B	exons 1-2	438	sense+antisense	lpy-dpy larvae (>80%)[e]
hlh1C	exons 4-6	299	sense+antisense	lpy-dpy larvae (>80%)[e]
hlh1D	intron 1	697	sense+antisense	wild type (<2% lpy-dpy)
myo-3 driven GFP transgenes [f]				
myo-3::NLS::gfp::lacZ			**makes nuclear GFP in body muscle**	
gfpG	exons 2-5	730	sense	nuclear GFP-LacZ pattern of parent strain
			antisense	nuclear GFP-LacZ pattern of parent strain
			sense+antisense	nuclear GFP-LacZ absent in 98% of cells
lacZL	exon 12-14	830	sense+antisense	nuclear GFP-LacZ absent in >95% of cells
myo-3::MtLS::gfp			**makes mitochondrial GFP in body muscle**	
gfpG	exons 2-5	730	sense	mitochondrial GFP pattern of parent strain
			antisense	mitochondrial GFP pattern of parent strain
			sense+antisense	mitochondrial GFP absent in 98% of cells
lacZL	exon 12-14	830	sense+antisense	mitochondrial GFP pattern of parent strain

Legend of Table 1

Each RNA was injected into 6-10 adult hermaphrodites ($0.5\text{-}1 \times 10^6$ molecules into each gonad arm). After 4-6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20-22 hours. Progeny phenotypes were scored upon hatching and subsequently at 12-24 hour intervals.

a: To obtain a semi-quantitative assessment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations. At the highest dose tested (3.6×10^6 molecules per gonad), the

individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-*unc22A* RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-*unc22A* RNA produced visible twitching in 30% of progeny.

b: *unc22C* also carries the intervening intron (43 nt).

c: *fem1A* also carries a portion (131 nt) of intron 10.

d: Animals in the first affected broods (laid at 4-24 hours after injection) showed movement defects indistinguishable from those of null mutants in *unc-54*. A variable fraction of these animals (25-75%) failed to lay eggs (another phenotype of *unc-54* null mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indicate partial inhibition of *unc-54* activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

e: Phenotypes of *hlh-1* inhibitory RNA include arrested embryos and partially elongated L1 larvae (the *hlh-1* null phenotype) seen in virtually all progeny from injection of ds-*hlh1A* and about half of the affected animals from ds-*hlh1B* and ds-*hlh1C*) and a set of less severe defects (seen with the remainder of the animals from ds-*hlh1B* and ds-*hlh1C*). The less severe phenotypes are characteristic of partial loss of function for *hlh-1*.

f: The host for these injections, PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ. This allows simultaneous assay for inhibition of *gfp* (loss of all fluorescence) and *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-*gfpG* caused a loss of GFP in all but 0-3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0-5 additional bodywall muscles and in the eight vulval muscles.

Table 2. Effect of injection point on genetic inhibition in injected animals and their progeny.

dsRNA	Site of injection	Injected animal phenotype	Progeny Phenotype
None	gonad or body cavity	no twitching	no twitching
None	gonad or body cavity	strong nuclear & mitochondrial GFP	strong nuclear & mitochondrial GFP
unc22B	Gonad	weak twitchers	strong twitchers
unc22B	Body Cavity Head	weak twitchers	strong twitchers
unc22B	Body Cavity Tail	weak twitchers	strong twitchers
gfpG	Gonad	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
gfpG	Body Cavity Tail	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
lacZL	Gonad	lower nuclear GFP	rare or absent nuclear GFP
lacZL	Body Cavity Tail	lower nuclear GFP	rare or absent nuclear GFP

Table 3. *C. elegans* can respond in a gene-specific manner to environmental dsRNA.

Bacterial Food	Movement	Germline Phenotype	GFP-Transgene Expression
BL21(DE3)	0% twitch	< 1% female	< 1% faint GFP
BL21(DE3) [*fem-1* dsRNA]	0% twitch	43% female	< 1% faint GFP
BL21(DE3) [*unc22* dsRNA]	85% twitch	< 1% female	< 1% faint GFP
BL21(DE3) [*gfp* dsRNA]	0% twitch	< 1% female	12% faint GFP

Table 4. Effects of bathing *C. elegans* in a solution containing dsRNA.

dsRNA	Biological Effect
unc-22	Twitching (similar to partial loss of *unc-22* function)
pos-1	Embryonic arrest (similar to loss of *pos-1* function)
sqt-3	Shortened body (Dpy) (similar to partial loss of *sqt-3* function)

In Table 2, gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence), and *unc-22* (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistence of products already present in the injected adult. After ds-*unc22B* injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with ds-*unc22A*. Injections of ds-*gfpG* or ds-*lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of ds-*gfpG* and ds-*lacZL* produced no twitching, while injections of ds-*unc22A* produced no change in GFP fluorescence pattern.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

WE CLAIM:

1. A method to inhibit expression of a target gene in a cell comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA comprises a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.

2. The method of claim 1 in which the target gene is a cellular gene.

3. The method of claim 1 in which the target gene is an endogenous gene.

4. The method of claim 1 in which the target gene is a transgene.

5. The method of claim 1 in which the target gene is a viral gene.

6. The method of claim 1 in which the cell is from an animal.

7. The method of claim 1 in which the cell is from a plant.

8. The method of claim 6 in which the cell is from an invertebrate animal.

9. The method of claim 8 in which the cell is from a nematode.

10. The method of claim 1 in which the identical nucleotide sequence is at least 50 bases in length.

11. The method of claim 1 in which the target gene expression is inhibited by at least 10%.

12. The method of claim 1 in which the cell is present in an organism and inhibition of target gene expression demonstrates a loss-of function phenotype.

13. The method of claim 1 in which the RNA comprises one strand which is self-complementary .

14. The method of claim 1 in which the RNA comprises two separate complementary strands.

15. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation outside the cell.

16. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation inside the cell.

17. The method of claim 1 in which the cell is present in an organism, and the RNA is introduced within a body cavity of the organism and outside the cell.

18. The method of claim 1 in which the cell is present in an organism and the RNA is introduced by extracellular injection into the organism.

19. The method of claim 1 in which the cell is present in a first organism, and the RNA is introduced to the first organism by feeding a second, RNA-containing organism to the first organism.

20. The method of claim 19 in which the second organism is engineered to produce an RNA duplex.

21. The method of claim 1 in which an expression construct in the cell produces the RNA.

22. A method to inhibit expression of a target gene comprising:

(a) providing an organism containing a target cell, wherein the target cell contains the target gene and the target gene is expressed in the target cell;

(b) contacting a ribonucleic acid (RNA) with the organism, wherein the RNA is comprised of a double-stranded structure with duplexed ribonucleic acid strands and one of the strands is able to duplex with a portion of the target gene; and

(c) introducing the RNA into the target cell, thereby inhibiting expression of the target gene.

23. The method of claim 22 in which the organism is an animal.

24. The method of claim 22 in which the organism is a plant.

25. The method of claim 22 in which the organism is an invertebrate animal.

26. The method of claim 22 in which the organism is a nematode.

27. The method of claim 26 in which a formulation comprised of the RNA is applied on or adjacent to a plant, and disease associated with nematode infection of the plant is thereby reduced.

28. The method of claim 22 in which the identical nucleotide sequence is at least 50 nucleotides in length.

29. The method of claim 22 in which the expression of the target gene is inhibited by at least 10%.

30. The method of claim 22 in which the RNA is introduced within a body cavity of the organism and outside the target cell.

31. The method of claim 22 in which the RNA is introduced by extracellular injection into the organism.

32. The method of claim 22 in which the organism is contacted with the RNA by feeding the organism food containing the RNA.

33. The method of claim 32 in which a genetically-engineered host transcribing the RNA comprises the food.

34. The method of claim 22 in which at least one strand of the RNA is produced by transcription of an expression construct.

35. The method of claim 35 in which the organism is a nematode and the expression construct is contained in a plant, and disease associated with nematode infection of the plant is thereby reduced.

36. A cell containing an expression construct,

wherein the expression construct transcribes at least one ribonucleic acid (RNA) and the RNA forms a double-stranded structure with duplexed strands of ribonucleic acid,

whereby said cell contains the double-stranded RNA structure and is able to inhibit expression of a target gene when the RNA is contacted with an organism containing the target gene.

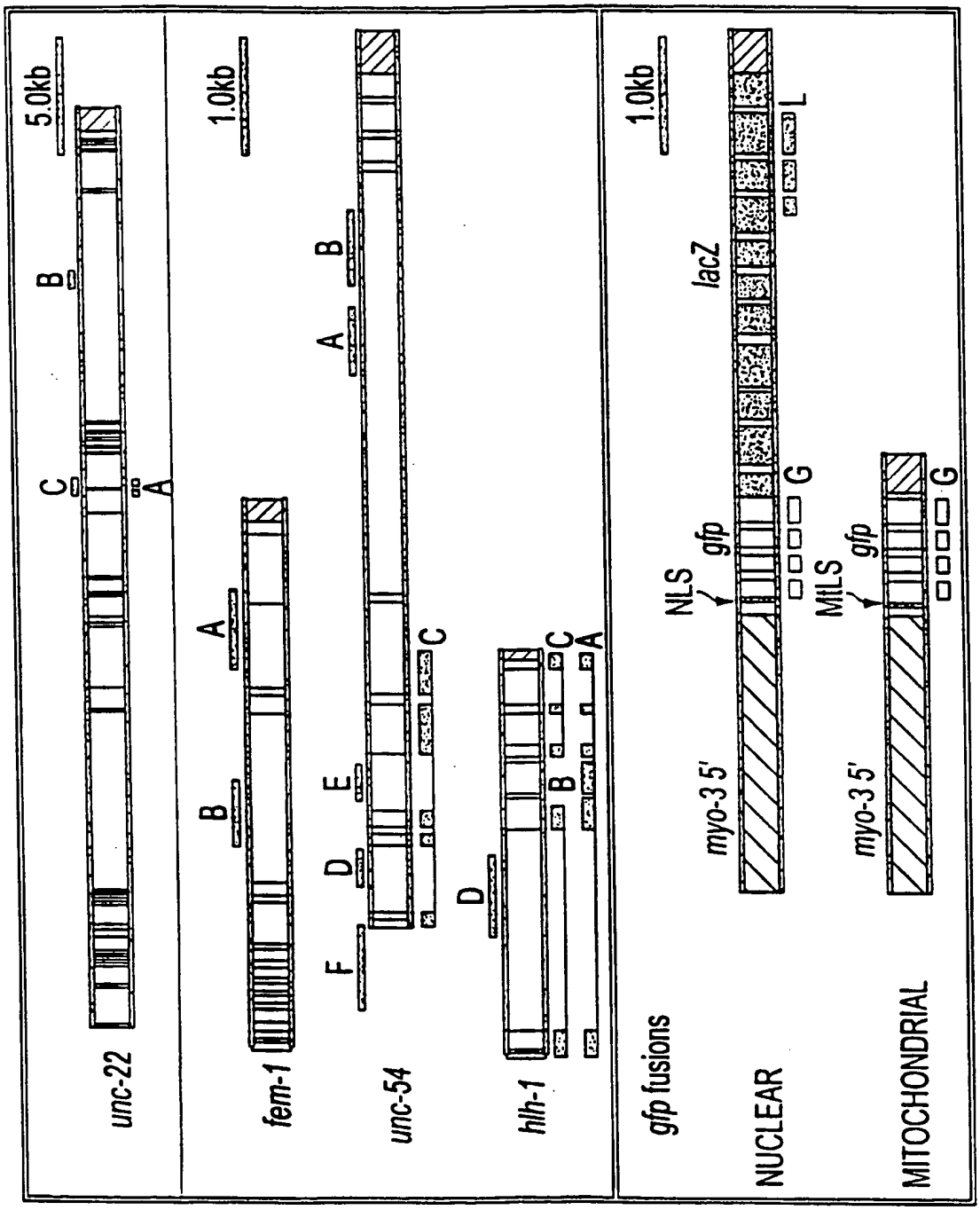
37. A transgenic animal containing said cell of claim 36.

38. A transgenic plant containing said cell of claim 36.

39. A kit comprising reagents for inhibiting expression of a target gene in a cell,

wherein said kit comprises a means for introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, and

wherein the RNA has a double-stranded structure with an identical nucleotide sequence as compared to a portion of the target gene.


5.0kb
C
B
A
unc-22
1.0kb
B
A
fem-1
A
B
F
D
E
C
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D
B
C
A
hlh-1
1.0kb
gfp fusions
NLS
gfp
myo-3 5'
lacZ
L
G
NUCLEAR
MtLS
gfp
myo-3 5'
G
MITOCHONDRIAL

FIG. 1

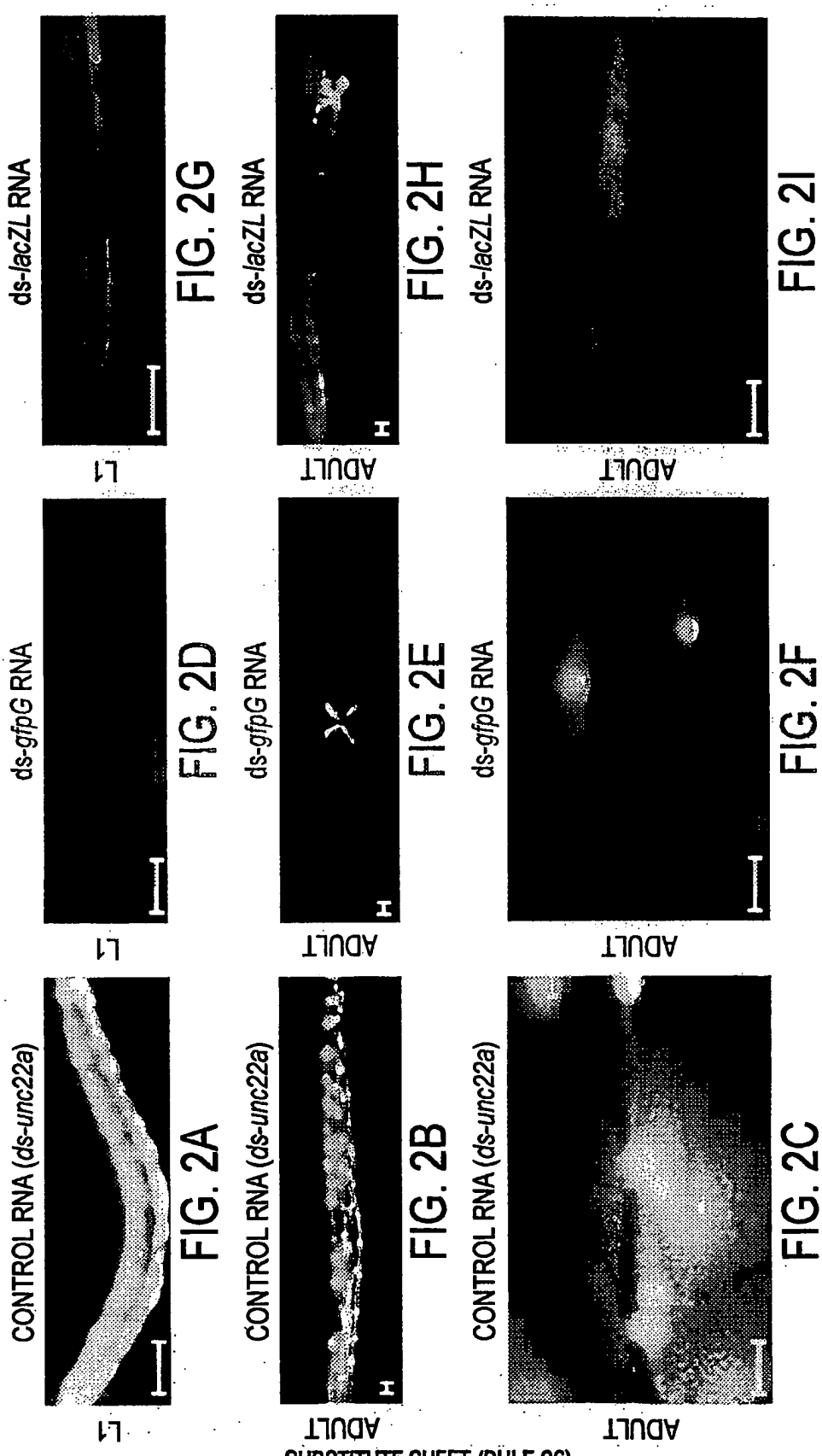

FIG. 2A FIG. 2B FIG. 2C
FIG. 2D FIG. 2E FIG. 2F
FIG. 2G FIG. 2H FIG. 2I

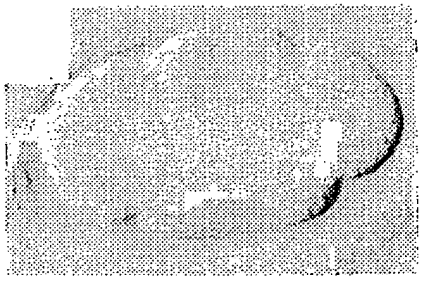

FIG. 3A

FIG. 3B

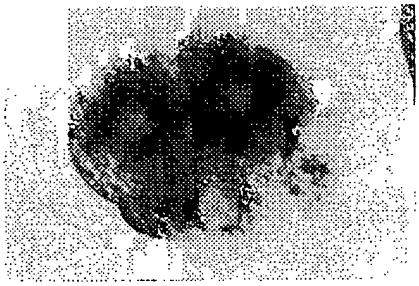

FIG. 3C

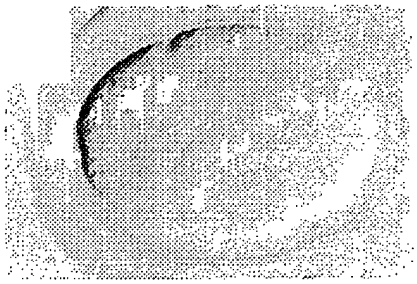

FIG. 3D

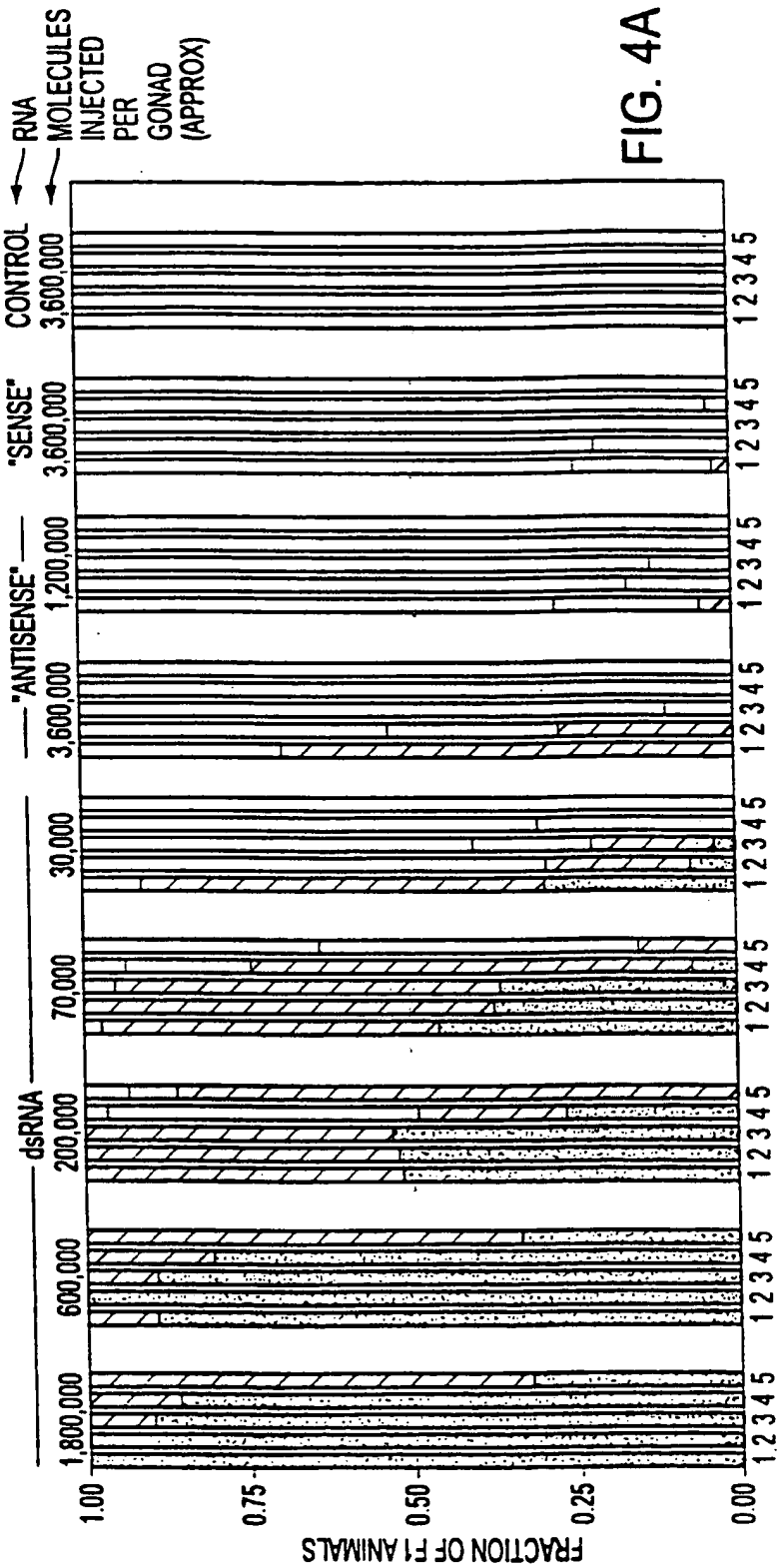
RNA MOLECULES INJECTED PER GONAD (APPROX)
CONTROL
"SENSE"
"ANTISENSE"
dsRNA
1,800,000
600,000
200,000
70,000
30,000
3,600,000
1,200,000
3,600,000
3,600,000
1 2 3 4 5
1.00
0.75
0.50
0.25
0.00
FRACTION OF F1 ANIMALS

FIG. 4A

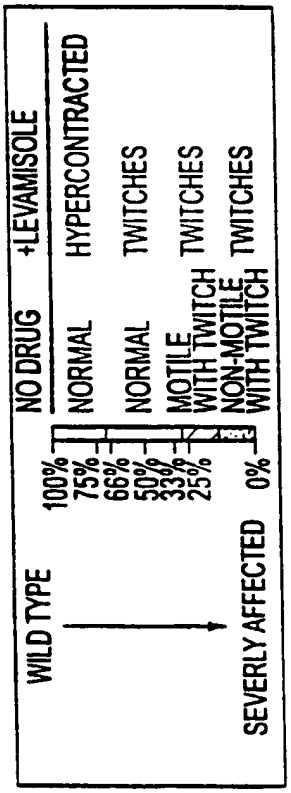
NO DRUG
+LEVAMISOLE
WILD TYPE
100%
75%
66%
50%
33%
25%
0%
NORMAL
HYPERCONTRACTED
NORMAL
TWITCHES
MOTILE WITH TWITCH
TWITCHES
NON-MOTILE WITH TWITCH
TWITCHES
SEVERLY AFFECTED

FIG. 4B

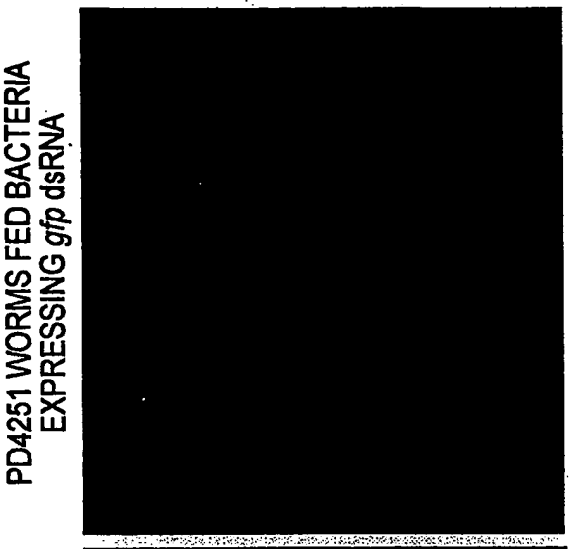

FIG. 5C

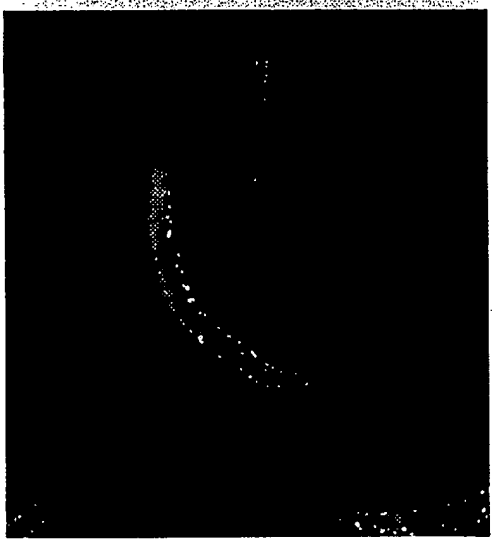

FIG. 5B

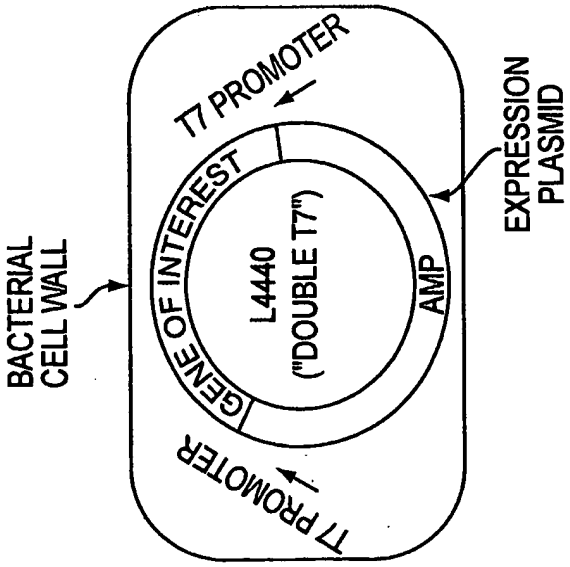

FIG. 5A

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 98/27233

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C12N15/63 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RATCLIFF F ET AL: "A similarity between viral defense and gene silencing in plants" SCIENCE, vol. 276, no. 93, 6 June 1997, pages 1558-1560, XP002095874 see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 May 1999	10/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ANDRES, S

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/27233

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	FIRE, A. ET AL.: "Production of antisense RNA leads to effective and specific inhibition of gene expression in C. elegans muscle" DEVELOPMENT (CAMBRIDGE, UK) (1991), 113(2), 503-14, XP002103600 cited in the application see page 508, right-hand column, paragraph 2 see page 509, right-hand column - page 511, right-hand column see page 512, 'Discussion' and figure 7 ---	1-39
A	MATZKE M A ET AL: "HOW AND WHY DO PLANTS INACTIVATE HOMOLOGOUS (TRANS)GENES?" PLANT PHYSIOLOGY, vol. 107, no. 3, 1 March 1995, pages 679-685, XP002021174 see page 680, left-hand column, paragraph 3 - right-hand column, paragraph 1 see page 682 ---	1
P,X	FIRE A ET AL: "Potent and specific genetic interference by double - stranded RNA in Caenorhabditis elegans" NATURE, (1998 FEB 19) 391 (6669) 806-11., XP002095876 cited in the application see the whole document ---	1-3,6, 8-12, 14-18, 21-23, 25,26, 28-31, 34,39
P,X	MONTGOMERY M K ET AL: "Double - stranded RNA as a mediator in sequence-specific genetic silencing and co - suppression" TRENDS IN GENETICS, (1998 JUL) 14 (7) 255-8., XP004124680 cited in the application see the whole document ---	1-4, 6-12, 14-18, 36-39
P,X	TIMMONS L ET AL: "Specific interference by ingested dsRNA" NATURE, (1998 OCT 29) 395 (6705) 854., XP002103601 cited in the application see the whole document -----	1-3,6, 8-12, 14-23, 25,26, 28-34, 36,39

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INTERNATIONAL SEARCH REPORT

...ernational application No.
PCT/US 98/ 27233

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 35 and claims 1-6,8-23,25-26,34 (as far as in vivo methods practised on animals are concerned) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.